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17
982

Experimental Gerontology

VOLUME 17 INDEX

1982

PERGAMON PRESS

New York Oxford Toronto Paris Frankfurt Sydney

Experimental Gerontology

VOL.
17
1982

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Published bimonthly.
For libraries, university departments, government laboratories, industrial and other multiple-reader institutions U.S. $180.00 (including postage and insurance). 2 year rate U.S. $342.00.
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Experimental Gerontology, Vol. 17, pp. 1–6, 1982
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17
982

FLUORESCENT AGE PIGMENT IN INSECT LYSOSOMES

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(*Received* 22 *February* 1981)

INTRODUCTION

FLUORESCENT MATERIAL which absorbs light in the range of 340–380 nm and emits near 450 nm has been associated with aged organisms, oxidative stress, and antioxidant deficiency. It has been termed wear and tear pigment, senile or age pigment, but most commonly lipofuscin. Its nature and origin are not yet perfectly clear. It is derived from peroxidation of polyunsaturated lipids (Strehler *et al.*, 1959). Chio (1969) has proposed that the pigment has a propeneimine structure, the propene portion coming from free radical peroxidative attack on polyunsaturated lipids, fragments of which combine with available amines in the cell to form the Schiff base. Support for the peroxidative mechanism was found in the fact that the antioxidant vitamin E inhibits formation of age pigment (Draper *et al.*, 1967).

Insects are good organisms for aging studies because of their short life cycles. Houseflies, fruit flies and honeybees are especially useful because of their rapid oxygen use during flight (Sohal, 1978; Sheldahl, 1974; Young, 1978). In addition, during insect metamorphosis, cells and cell components are broken down for synthesis of new tissue. Lysosomal enzymes are increasingly active at this time and the electron dense material which accumulates in the lysosomes may be related to age pigment (Young, 1979).

I have used lysosomes from the larval midgut of the southern armyworm, *Spodoptera eridania* (Cramer) to determine amounts of fluorescent pigment and to gain some knowledge of its nature and similarity to other well studied age related material.

MATERIALS AND METHODS

Chemicals

Solvents were reagent or spectral grade. Each batch was tested for fluorescence and glass distilled if necessary. Diethyl ether was dried over $CaCl_2$ and distilled from sodium metal.

Insects

Larvae of the southern armyworm *Spodoptera eridania* were reared on bean plants in a greenhouse through

five larval stadia (Krieger and Wilkinson, 1969). The sixth instars were kept in an environmental chamber at 27°C, 80% RH and 16/8 light/dark period. After about 80 hours the larvae burrow and become pupae (old sixth instars). At this stage when the guts were visually disintegrating, the lysosomes were isolated.

Some comparisons of the pigment were made with lysosomes of larvae which had entered the 6th stadium within 12 hours (young sixth instars).

Tissue preparation

The midgut sections were homogenized with a Potter Elvejhem type homogenizer in cold 0.25 M sucrose. The Teflon ® pestle clearance was 0.005" and 12 passes were made. The homogenate was fractionated by the method of Ragab *et al.* (1967) to yield the lysosome composite fraction which was suspended in water.

Suspensions of lysosomes containing 3–6 mg protein in 2.0 ml H_2O were extracted for one minute with 3.0 ml of a 2:1 mixture of chloroform methanol with a vortex mixer. After centrifugation, the lower chloroform layer was washed with water, centrifuged and the fluorescence of the chloroform layer measured. A second extraction yielded negligible fluorescence and no advantage was noted by extracting in a nitrogen atmosphere. When needed for other experiments the chloroform extracts were dried under nitrogen and so stored at −18°C.

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Analysis

Fluorescent properties of extracts were examined in an Aminco SP 125 spectrofluorometer with a 1P 28 photomultiplier (S-5 response). The excitation and emission slits were 2 and 1 mm respectively. Several dilutions of the extracts were made to avert quenching errors.

To allow comparison of different preparations of lysosomal extracts, the instrument was calibrated daily with a solution of 0.1 μg/ml quinine sulfate in 0.1 N H_2SO_4. It was set to read at 9 on the 0.3 × full scale. Fluorescence of chloroform extracts read at 360 ex/430 em were compared to quinine sulfate on the basis of the lysosomal protein used for the extraction. The values found were usually in the range of $2\text{–}10 \times 10^{-3}$.

Protein was measured by the Lowry method (1951), phosphorus by that of Fiske Subba Row (1925), and total nitrogen by Nessler reagent after wet washing with H_2SO_4. Thiobarbituric acid (TBA) reactive materials were assayed by heating water suspensions of lysosomes at 100°C for one hour in 0.14 M thiobarbituric acid in phosphate buffer pH 6.0. The absorbance was read at 532 nm. Standard was malonaldehyde tetramethylacetal.

Glutathione peroxide was assayed by the method described by Chow *et al.* (1970).

Measures of diene conjugation in extracts of lysosomes were obtained by absorbance measurements at 237 nm (May, 1973).

Chromatography

Silicic acid (Bio.-Sil A, Bio Rad Laboratories, Richmond, CA) was used for column chromatography. Columns 8 × 160 mm were conditioned according to Hirsch and Ahrens (1958). The elution protocol was that of Bjorkerud (1964).

Thin layer plates without fluorescence indicator (Whatman LK5D ®) were washed with chloroform, methanol, acetic acid 75:25:1; before activation at 110°C. After development, the plates were examined for fluorescence under a mercury lamp with a Woods filter. The methodology of Dittmer and Lester (1964) was used for lipid detection.

RESULTS AND DISCUSSION

The fluorescence spectrum of the gut lysosome chloroform extract is shown in Fig. 1. It is similar to the previously described age related pigment in that the emission was decreased by alkali and destroyed by ultraviolet light (Malshet *et al.*, 1974).

When the aqueous lysosomal preparation was treated with a peroxidation mixture and oxygen (Dillard, 1973) the results shown in Fig. 2 were obtained. These indicate that the fluorescent material resulted from lipid peroxidation which also yields thiobarbituric acid reactive fragments.

Two experiments comparing old larvae (about to pupate) with young sixth instars were done, one compared a total of 21 lysosome preparations (1,500 insects) and another with 12 preparations (900 insects). The older insects accumulated more pigment in lysosomes. The statical probabilities of real differences were 80% (*t*-test).

The elution pattern shown in Fig. 3 was typical of the material making up the lipids of

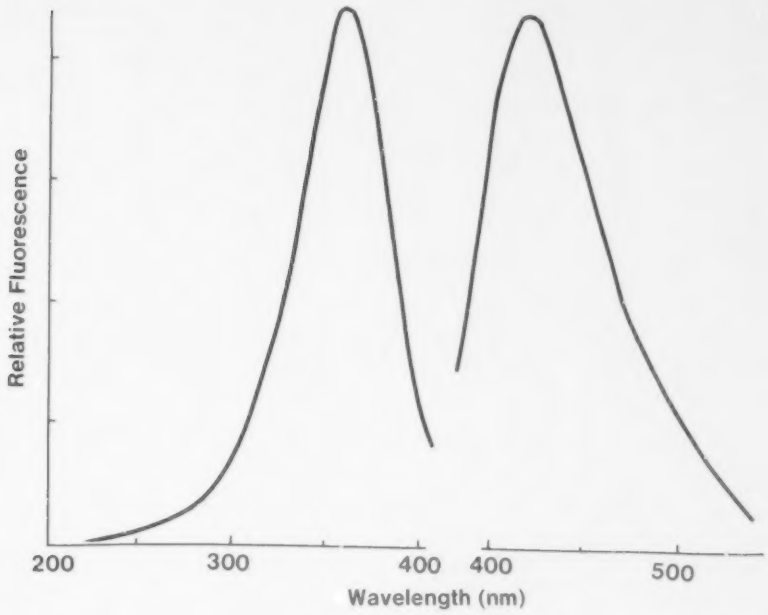

FIG. 1. Fluorescence spectrum of chloroform extract of *Spodoptera* gut lysosomes.

the prepupal lysosomes. The fluorescent material was displaced sharply from the silicic acid by solvent VI, the 9:1 mixture of chloroform methanol. A run with the extract from early sixth instars yielded a 10% smaller fluorescent fraction. In each case the fluorescent spectra were identical to that of the chloroform extract.

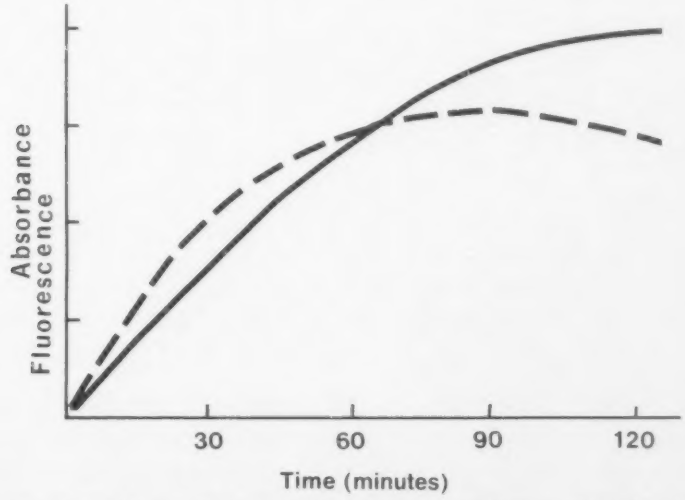

FIG. 2. Solid line = Fluorescence 360/430 nm; Dashed line = Thiobarbituric acid reaction 532 nm.

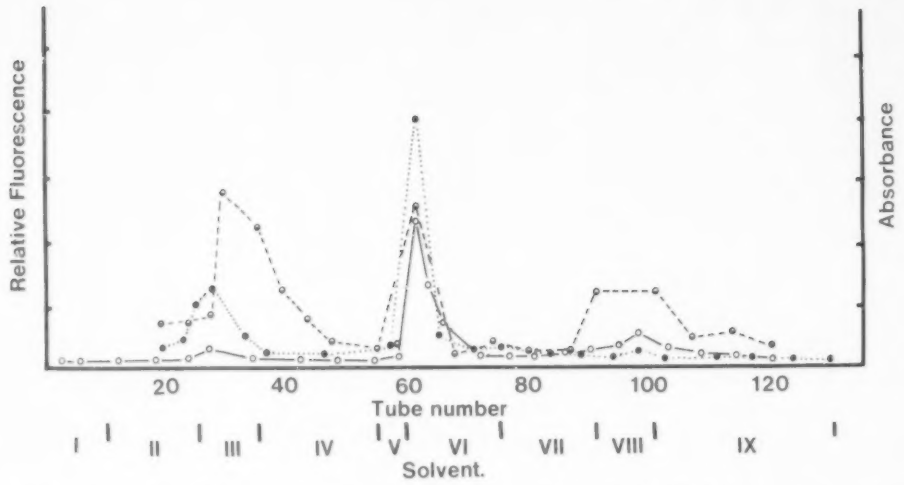

FIG. 3. Solvent: I = 4% ether in hexane; II = 8% in hexane; III = 25% ether in hexane; IV = ether; V = chloroform; VI = chloroform 9, methanol 1; VII = chloroform 3, methanol 2; VIII = chloroform 1, methanol 1; IX = chloroform 1, methanol 4. Tubes contained 1.2 ml. o————o = Fluorescence 360/430; o— — —o Absorbance 532 nm (TBA reaction); •·····• Absorbance 237 nm (diene conjugation).

The principal material which reacts with thiobarbituric acid (TBA) is considered to be malonaldehyde (Dahle *et al.*, 1962). Its production by reaction with polyunsaturated lipids was evident in tubes which contained triglyceride and phospholipid, mainly eluted by solvents III, IV and VIII, IX respectively. Only in fractions from solvent VI were TBA reactivity and fluorescence evident. Additionally, the same fraction had a high level of conjugated unsaturation. Eighty percent of *Spodoptera* gut lysosome fatty acids are unsaturated (Young, unpublished).

The fluorescent fraction does not appear to be phospholipid. The chromatograms in Fig. 4 indicated it to be less polar than phospholipid and the presence of only 0.15% phosphorus made it more unlikely. Nesslers reagent failed to detect nitrogen in the wet ashed fluorescent eluate from the silicic acid column.

Privett *et al.* (1955) reported on the complex reactions of unsaturated lipids with oxygen. The products investigated included conjugated diene, peroxides, hydroperoxides and polymers. The fluorescent material here has ultraviolet absorption typical of conjugated dienes, and the presence of thiobarbituric acid reacting material is consistent with oxygen functions such as hydroperoxides.

The small amounts of fluorescent material available for lysosomes, (about 3,000 worms were used for tests for phosphorus and nitrogen in the fluorescing fraction) made characterization difficult.

Mass spectral and infrared analysis were not definitive and it is probable that the fraction was not homogeneous. I suggest that it is a mixture of partially degraded lipids and/or polymerized lipid fragments which have accumulated in the lysosomes. It is evident that the accumulation begins in the early part of larval life just as lipofuscin is found in young as well as old organisms (Kent, 1967).

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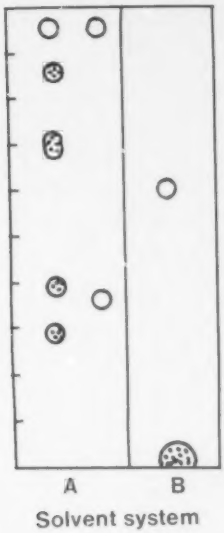

FIG. 4. A. Chloroform 15.4, methanol 5.6, ethanol 2.0, H_2O 1.0. Left track = Lysosome extract; Right track = $CHCl_3$ extract of reaction of malonaldehyde; with cyclohexylamine (upper) and glycine (lower) (Chio, 1969). B. Hexane 50, Diethyl Ether 50, acetic acid 2; Lysosome extract; Open circles = blue fluorescence; Stippled circles = phospholipid stain = molybdic acid.

It was not possible to evaluate the effect of antioxidants on the insects. The plant diet is well supplied with vitamin E. The protective effect against peroxidation by the enzyme glutathione peroxidase in lysosomes is minimal. It was detectable only with long incubation times.

SUMMARY

Fluorescent pigment accumulates in the lysomal fraction of *Spodoptera* sixth instars. It was purified by silicic acid chromatography. The fluorescent fraction was characterized by conjugated unsaturation, reactivity with thiobarbituric acid, and lability in alkali; all reminiscent of age pigment. The phosphorus content of the purified fraction was negligible and nitrogen was not detected. It is suggested that the material is degraded, perhaps polymerized lipid fragments.

Acknowledgement—I gratefully acknowledge the technical assistance of Marie Rotter and Rosemary Hunter.

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Experimental Gerontology, Vol. 17, pp. 7–9, 1982
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AGE-RELATED CHANGES IN PYRIDINOLINE CONTENT OF RABBIT COLLAGEN

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COLLAGEN HAS BEEN shown to play a major role in the aging process in man and animals (Sinex, 1968). Relative increase in collagenous proteins and a decrease in myosin and total proteins in muscular atrophy has been suggested by various authors (Fisher and Ramsey, 1946; Helander, 1958; Guth, 1969). It is generally accepted that collagen fibers increase in stability to external influences with increasing age (Gallop *et al.*, 1972). These changes may be related to the content and chemical nature of the covalent cross-links between collagen molecules (Gallop *et al.*, 1972). Although structures of several cross-links have been determined (Bailey and Shimokomaki, 1971; Tanzer, 1973), they are too labile to acid and heat to account for mature collagen fibers and they disappear by the time the animals have reached maturity (Fujii and Tanzer, 1974; Fujiimoto *et al.*, 1977). It has been suggested that the labile cross-links are converted to pyridinoline, a more stable cross-links during maturation (Fujiimoto *et al.*, 1977; Fujiimoto and Moriguchi, 1978). However, the content of pyridinoline from rabbit collagen during aging with reference to red, white and cardiac muscles have not been reported so far, and hence the present study.

MATERIALS AND METHODS

Samples of red, white and cardiac muscles were obtained from freshly killed rabbits of ages varying from 2 to 45 weeks. Each sample was cleaned of adhering tissue, sliced into small pieces, soaked in 0.5 M NaCl for 24 h at 4°C and washed with several changes of cold water. The sample (about 500 mg wet weight) was then hydrolysed with 6N HCL (5ml) at 110°C for 24 h in sealed tube. The hydrolysate was evaporated and the residue was taken up in water (5ml). A sample (2–4 ml) was applied to a P-cellulose column (H^+ form, 1.2 × 8 cm). Elution was performed using a linear gradient formed from 50 ml of water and 50 ml of 0.5 N HCL. The fraction (3 ml each) were collected and the absorbance at 295 nm was recorded. Pyridinoline was usually eluted between fraction numbers 26 and 29. These fractions were pooled and the fluoroscence at 395 nm was measured with excitation at 295 nm using a fluoroscence spectrophotometer (Fujiimoto and Moriguchi, 1978). The concentration of pyridinoline was estimated by comparision of its fluoroscence intensity with that of a known amount of pure pyridinoline (a gift from Dr. D. Fujiimoto). Another portion of the hydrolysate was assayed for hydroxyproline according to the method of Kivirikko *et al.* (1967) and the amount of total amino acid in the collagen was estimated from its hydroxyproline content to be 95 residues per 1000 total residues.

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RESULTS AND DISCUSSION

The age-related changes in the pyridinoline content of rabbit muscle collagen are shown in Table 1. The pyridinoline content of collagens in 2-week-old animals was very low. It increased markedly with growth of the animals. This marked increase was completed by the time the animals reached physiological maturity, i.e., about 21 weeks rabbits tissues. These changes appear to be concomitant with the decrease in the content of aldehyde, the labile cross-links and with the increase in mechanical strength of collagen fibers (unpublished data). The result suggests that pyridinoline is a mature cross-links of collagen and its formation might be an essential step in the growth process to obtain normal mechanical strength in the collagen fibers.

The changes in the content of pyridinoline after maturity varied with tissues. In the case of the red and white muscles, the pyridinoline continued to increase gradually up to 45 weeks. On the other hand the pyridinoline content began to decrease after 21 weeks of age in the case of cardiac muscle.

This result suggests that, in the cardic muscle of rabbit, pyridinoline is gradually transformed to some other compounds. The mechanism of the alteration, the nature of the product and the physiological significance remain to be elucidated. However, pyridinoline may serve as an interesting index for both the maturation and senescence of the cardiac muscle. In the red & white muscle of old rabbits such alteration of pyridinoline apparently did not occur. The reason and physiological significance for the difference between red & white muscle and cardiac muscle remain to be elucidated.

SUMMARY

The age related changes in the content of pyridinoline were followed for collagen from rabbit muscles. Pyridinoline content of the muscle collagen in 2-week-old rabbits is very low and increases markedly with growth of the animals. However, it decreases after maturation in cardiac muscle but not in red & white muscle. Pyridinoline may serve as an interesting index for aging in connective tissue.

Acknowledgments—I am thankful to Professor D. Fujiimoto, Department of Chemistry, Hamamatsu University School of Medicine, Hamamatsu, Japan for his generous gift of pure pyridinoline, and Professor G.S. Ghosh, G.M. College, Sambalpur for providing necessary facilities.

TABLE 1. PYRIDINOLINE CONTENT* IN COLLAGEN FROM RABBIT MUSCLE

Age (weeks)	Red muscle	White muscle	Cardiac muscle
2	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
12	0.07 ± 0.01**	0.08 ± 0.01**	0.07 ± 0.01**
21	0.10 ± 0.03**	0.11 ± 0.02**	0.13 ± 0.03**
32	0.11 ± 0.01	0.11 ± 0.03	0.09 ± 0.01**
45	0.11 ± 0.02	0.12 ± 0.01	0.08 ± 0.02

*Pyridinoline content is expressed in residues per 1,000 total amino acid residues. Results are expressed as mean of six samples ± standard deviation.

**Significantly different from preceding value in same column ($p < 0.05$).

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Experimental Gerontology, Vol. 17, pp. 11–17, 1982
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0531-5565/82/010011-07$03.00/0
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AGE-RELATED DNA FRAGMENTATION IN TWO VARIETIES OF *DROSOPHILA MELANOGASTER, PHASEOLUS* (COTYLEDONS), AND THREE TISSUES OF THE MOUSE

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(*Received* 12 *March* 1981)

INTRODUCTION

In a review, Hahn (1970) stated that evidence for aging mechanisms at the chromatin level come from two types of studies. The first approach is to examine the structural integrity of the DNA, while the second would be to examine functional aspects of the molecule, such as ability to serve as a template. Structural changes to the DNA of an organism could adversely affect its ability to survive.

The possibility of DNA degradation being an important factor in senescence is raised by the reports of Massie *et al.* (1972) and Ono *et al.* (1976). Massie *et al.* (1972) reported an approximately 10-fold decrease in the molecular weight of both single- and double-strand DNA from rat liver. They indicated that most of the decrease occurred in animals less than 400 days old (13–14 months). DNA isolated from mouse liver was also found to become fragmented by 14 months of age (Ono *et al.*, 1976). No further decreases in molecular size were noted beyond 14 months, and no evidence of fragmentation was found in DNA from spleen, thymus, and brain. In contrast, no evidence of DNA fragmentation was found in wild-type *Drosophila* (Massie *et al.*, 1975).

Therefore, experiments were undertaken to determine if DNA fragmentation with age was confined to the liver of small mammals or if it occurred in other aged organisms as well.

MATERIALS AND METHODS

Culture of organisms

Drosophila melanogaster (Oregon-R and vestigial wing strains) were cultured on a corn meal-molasses-agar medium (Samis *et al.*, 1971) in 30 ml clear plastic vials having plastic foam stoppers. Cultures were housed in a Freas 815 low temperature incubator at 20 ± 0.5°C. Flies were transferred weekly to fresh culture media.

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Phaseolus vulgaris seeds were imbibed overnight and planted in vermiculite. Seedlings were grown in a climate controlled plant growth room, daytime temperature 72°F, nighttime temperature 66°F, with a 16 h light, 8 h dark photo period. They were watered daily with 1 tsp of 8% nitrogen, 8% phosphoric acid, 20% potash per gallon of water. C57Bl/6J female retired breeder mice (The Jackson Laboratories, Bar Harbor, Maine) were housed in polycarbonate cages with wire lids and a molded spun polyester filter top in an animal room with a 12 h light, 12 h dark photo period. Mice were fed and watered *ad libitum*. Cages were cleaned thrice weekly with minimal handling. Mice were killed by cervical dislocation.

DNA extraction

Organisms and tissues were homogenized in 5 volumes (w/v) of buffer (0.05M TRIS, 0.025 M KCl, 0.005 M $MgC_2H_3O_2$, 0.35 M sucrose; pH 7.6) (Ritossa and Spiegelman, 1965). For the mouse tissue and *Drosophila*, homogenization was done using a glass homogenizer with a motor driven Teflon ® pestle. *Phaseolus* cotyledons were homogenized using a Polytron homogenizer (Brinkman Instruments) at setting 9. The homogenate was filtered through cheese cloth; SDS was added to a final concentration of 2% (5% for the mouse tissues) followed by shaking in a 60°C waterbath for 30 min. After adding an equal volume of cold 95% ethanol, the precipitate was centrifuged in a Sorvall RC2-B (SS − 34 rotor) for 10 min at 10,000 rpm. The pellet was resuspended in 10 ml of 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate; pH 7.), then adjusted to 1 × SSC. Deproteinization was done using Marmur's procedure (Marmur, 1961). Following this, RNA was removed by alkaline hydrolysis (Bock, 1967). The amounts of DNA isolated were determined by the A_{260} on a Beckman DB-GT recording spectrophotometer. Calculations were made using an extinction coefficient derived from Salmon DNA (Sigma).

Agarose gel electrophoresis

Analytical 0.5% (w/v) Agarose (Bio Rad Laboratories) gels were prepared by melting the agarose in TBE buffer (0.089 M TRIS, 0.089 M Boric Acid, 2.5 mM EDTA; pH 8.5) (Sugden *et al.*, 1975). Ethidium bromide (Sigma) was added to a final concentration of 1.0 μg/ml. Gels were made in a 10.5 × 22.0 cm Model 4006 acrylic agarose gel bridge (Bio Rad Laboratories) using a 5-slit forming polycarbonate comb for the sample well formation. DNA samples were made 10% with glycerol and layered into the sample wells which were previously filled with TBE-ethidium bromide buffer. The electrode chambers of a Bio Rad Model 1405 electrophoresis cells were filled with TBE-ethidium bromide buffer, and electrophoresis was carried out at 5 volts/cm for 8 h using a Bio Rad Model 1420 power supply. A six-fragment DNA molecular weight marker (Boehringer Mannheim) was used in one of the sample wells for comparison (Thomas and Davis, 1975).

RESULTS

Life spans of Drosophila *strains*

The survival curves of the two *Drosophila* strains, under the conditions of this study, are presented in Fig. 1. The mean life span of the wild-type (Oregon-R) strain was 43.2 days, while that of the vestigial wing mutant was 27.3 days. These mean life spans are somewhat longer than those previously reported (Gonzalez, 1923; Biscardi and Webster, 1977). However, these flies were cultured on a more defined medium and were kept approximately 5°C cooler, which may account for the increased survival and the lessened slope of the survival curve when compared to Biscardi and Webster (1977).

Agarose gel electrophoresis

Agarose gel electrophoresis has been shown to be quite useful in the separation of DNA fragments by molecular size (Sugden *et al.*, 1975; Thomas and Davis, 1975). The results of the electrophoresis are illustrated in Figs. 2 through 5. DNA from Oregon-R and vestigial *Drosophila* showed no fragment formation (Fig. 2) with respect to age. *Phaseolus* cotyledon DNA (Fig. 3) showed migration of an approximately 3.5 megadalton fragment in both imbibed seed DNA and DNA from 12-day-old cotyledons, which by this time were yellowed and wrinkled, and in some cases had already fallen from the plant. Thus, there appeared to be no age-related change.

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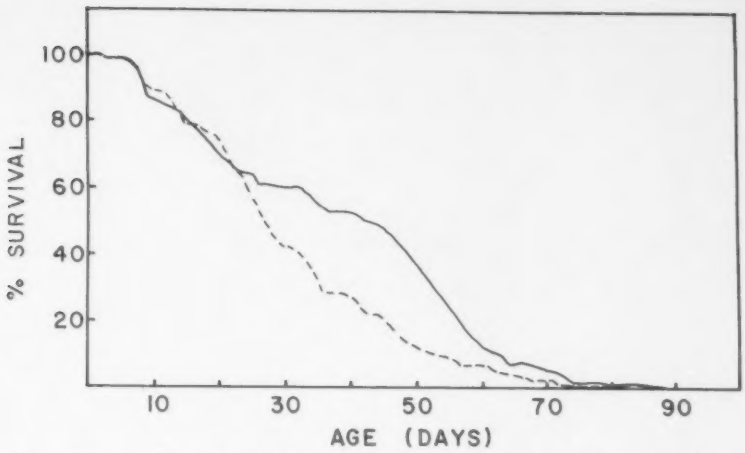

FIG. 1. Life spans of two strains of *Drosophila melanogaster* cultured as described at 20 ± 0.5°C, Oregon-R (———); Vestigial wing (----).

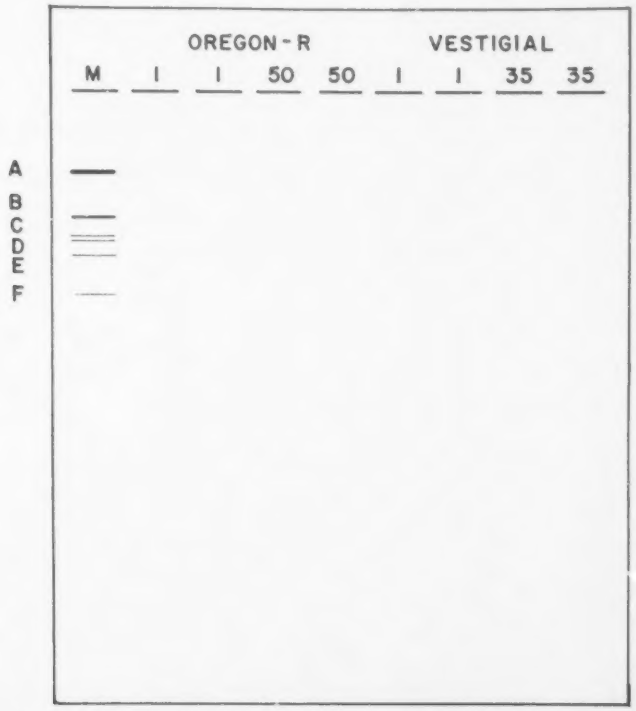

FIG. 2. Agarose gel electrophoresis results of DNA isolated from two strains of *Drosophila melanogaster*. M = marker DNA producing fragments A (13.7 megadaltons), B (4.74 megadaltons), C (3.73 megadaltons), D (3.48 megadaltons), E (3.02 megadaltons), and F (2.13 megadaltons). DNA was from Oregon-R of one- and 50-day old flies, while one- and 35-day old vestigial flies were used. Note that no fragmentation occurred in either strain at either age.

FIG. 3. Electrophoresis results of DNA from *Phaseolus* cotyledons. M = marker with fragments as described in Fig. 2. IS = imbibed seeds. 12 = 12-day old cotyledons. Note the presence of a fragment of approximately 3.5 megadaltons in both the IS and 12-day old samples.

In the mouse, DNA's from the brain and heart (Fig. 4a and 4b) showed no fragmentation in either 8-, 17-, or 24-month old tissues. However, liver DNA showed a large fragment (estimated at 24 megadaltons) in each of the three ages tested (Fig. 5a and 5b). Therefore no age-related changes were observed.

DISCUSSION

DNA isolated from two varieties of *Drosophila* showed no fragment formation with increasing age. This supports the findings of Massie *et al.* (1975) with regard to wild-type flies. Also, the vestigial wing mutant showed no fragment formation with age, even though its mean life span was about 36% less than wild-type.

It appeared that *Phaseolus* cotyledons normally possess a DNA fragment of approximately 3.5 megadaltons and that this fragment was not formed as a result of aging. The

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FIG. 4a. Electrophoresis results of DNA from mouse brain and heart. Representation of results of marker (M) and DNA from 8- and 17-month-old tissues. Note the absence of any fragmentation.

FIG. 4b. Electrophoresis results of DNA from mouse brain and heart. Photograph of gel obtained using DNA from 24-month-old tissues. Note the absence of any fragmentation.

results obtained from mouse liver agreed with both Massie *et al.* (1972) and Ono *et al.* (1976) in that some fragmentation occurs prior to 14 months of age, but does not change thereafter. It appeared that this fragmentation was unique to liver tissue, as none was found in brain and heart.

While fragmentation of DNA could have a profound effect on an organism's survival, it appears that this was not of universal occurrence with respect to aging. It seems that in mammalian systems, DNA fragmentation is unique to liver tissue and does not occur in post-mitotic tissues such as brain and heart, as well as in essentially post-mitotic organisms such as *Drosophila*.

SUMMARY

Agarose gel electrophoresis showed that no age-related DNA fragmentation occurred in wild-type or vestigial wing *Drosophila*, or in DNA from mouse brain and heart. DNA from mouse liver possessed a large fragment, estimated at 24 megadaltons, in all ages tested. *Phaseolus* cotyledon DNA possessed a fragment of approximately 3.5 megadal-

FIG. 5a. Electrophoresis results of DNA from mouse liver. Representation of results of marker (M) and DNA from 8- and 17-month-old tissues. Note that there is a band present just under the sample well, which is estimated to be approximately 24 megadaltons.

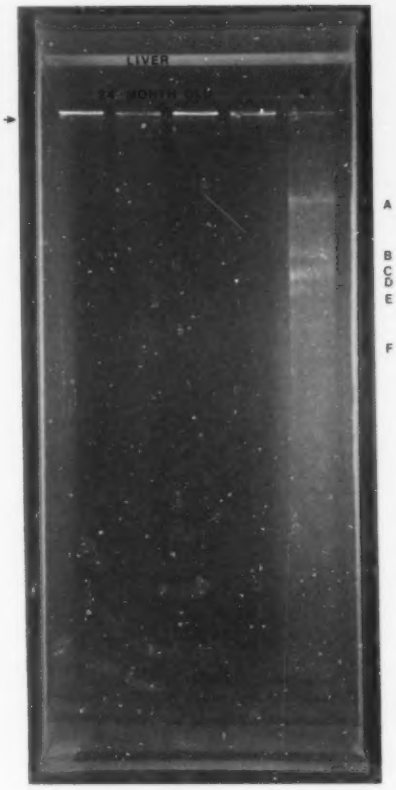

FIG. 5b. Electrophoresis results of DNA from mouse liver. Photograph of gel obtained using DNA from 24-month-old tissues. Note that there is a band present just under the sample well, which is estimated to be approximately 24 megadaltons.

tons in both imbibed seeds and 12-day-old cotyledons. Thus, there appeared to be no age-related increase in the break-down of DNA into large fragments.

Acknowledgement—This work was supported in part by U.S. Public Health Service grant S08 RR09032 awarded by the Biomedical Research Development Grant program, National Institutes of Health, and National Science Foundation grant SER-7706567.

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Experimental Gerontology, Vol. 17, pp. 19–31, 1982
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INFLUENCE OF AGE UPON THE CEREBRAL METABOLIC CHANGES INDUCED BY ACUTE HYPOXIA ON THE SYNAPTOSOMES FROM DOG BRAIN

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(*Received* 12 *March* 1981)

INTRODUCTION

The ageing of cerebral tissue has been studied by means of a series of models, using various biochemical and clinical techniques and evaluating different parameters (Sokoloff, 1979). Special attention has been focused on changes in oxygen consumption and cerebral blood flow in man, as a function both of age and of the various diseases connected with it, e.g., senile dementia (Lassen *et al.*, 1957) and late psychoses (Freyhan *et al.*, 1951). The phenomenon of ageing has been investigated also through tissular metabolic parameters, such as the content of intermediates and substrates of the various cerebral metabolic pathways (Ford, 1973; Maker *et al.*, 1973; Timiras *et al.*, 1973) as well as the activity of cerebral enzymes (Wilson, 1973), and as a function of different pharmacological treatments (Benzi, 1979; Benzi *et al.*, 1979a, 1980a). On the other hand, cerebral disorders can be classified (Meier-Ruge *et al.*, 1975) into cerebrovascular insufficiency and psychoorganic disease, the latter including metabolic homeostasis derangements. In particular, defects of maturity and senescence appear to be related to age-dependent adaptation failure (Meier-Ruge *et al.*, 1975, 1976). In effect, the brain vessels of young rats adapt to hypoxia and hypocapnia by modifying the local cerebral blood flow (LCBF) more rapidly than those of older rats, while no significant LCBF differences can be found between the two groups under basal conditions (Haining *et al.*, 1969). On the other hand, for many years the effect of drugs at cerebral level has been ascribed to their ability to affect the cerebral blood flow (CBF), metabolic effects being regarded as secondary to these modifications. Experimental studies with acute models have been carried out to detect possible pharmacological actions on cerebral metabolism related to energy transduction in normal, hypoxic or ischemic brains (Benzi *et al.*, 1973; Benzi, 1975; Benzi and Villa, 1976; Benzi *et al.*, 1977, 1980b).

In this work we studied the effects of age and of pharmacological treatment, both under

normal conditions and after 15 min of acute hypoxic hypoxia, on the glycolytic metabolites (pyruvate, lactate) and energy mediators (ATP, ADP, AMP and creatine phosphate) of the cerebral cortex (motor area) and of the synaptosomes obtained from the controlateral cortical motor area. These effects were evaluated in beagle dogs aged 1.5, 6.5 or 12 years ("young adult," "mature" and "senescent" dogs, respectively). In addition, oxygen consumption rate and cytochrome *a* and *c* content were measured in synaptosomes. The drugs used were chosen on account of their gross pharmacodynamic characteristics, including: (a) vascular action, i.e., papaverine; (b) vascular action associated with metabolic action, i.e., (-)eburnamonine and dihydroergocristine; (c) protective action against hypoxia- and ischemia-induced metabolic damage, i.e., phenobarbital.

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MATERIALS AND METHODS

The experiments were carried out on female beagle dogs aged 1.5 years: "young adult" dogs; or 6.5 years: "mature" dogs; or 12 years: "senescent" dogs. These attributions are totally arbitrary and imprecise. However, they are used to facilitate the singling out of given times during dogs' lives. The animals were maintained under standard environmental conditions (temperature = 22 ± 1°C; relative humidity = 60 ± 5%) and fed a standard diet as pellets, with water *ad libitum*. The surgical procedure was performed on animals starved overnight and preanesthetized with urethane (0.4 g/kg i.p.). Anesthesia was induced and maintained during the surgical procedure by chloralose (40 mg/kg i.v.) and by intermittent nitrous oxide inhalation. During the operative procedure and the experiment, the animals were treated with an i.v. injection of gallamine triethiodide (2–3 mg/kg) to permit unresisted artificial respiration and to maintain a constant $PaCO_2$. The dogs were artificially ventilated with air (normocapnic normoxic condition) or with a 95:5 nitrogen-oxygen mixture (normocapnic hypoxic condition). The changes in the energy charge of the brain induced by hypoxemia are magnified by the decrease in arterial blood pressure (Siesjö and Nilsson, 1971; Benzi and Villa, 1976; Benzi *et al.*, 1978). Therefore the experiments were carried out in hypovolaemic hypotension (MABP = 55 ± 4 mm Hg). The dogs were slowly bled within 15 min from an arterial femoral cannula into a heparinized bottle, with three openings to withdraw and oxygenate the blood from systemic circulation. The bottle was connected to a femoral vein to reinfuse (if necessary) the drained blood into the systemic circulation by means of a peristaltic pump.

The procedure (Benzi *et al.*, 1978) consisted mainly in isolating both common carotid arteries, while the zygomatic, maxillary, auricular, and supraorbital vessels were all occluded by ligature or compression. The head was fixed to a head-holder with the confluence of the cerebral venous sinuses approximately 10 cm higher than the heart. After bilateral longitudinal incisions of skin and muscles, two 2.5–3.5 cm diameter holes were made in the frontal parietal bones of the skull. A plastic funnel was fitted into each hole and both muscle and skin were sutured tightly around the funnels. These were sealed with a rubber stopper and insulated to prevent untoward ambient heat and pressure exchange. Both femoral arteries were cannulated: one connected to a physiological pressure transducer and the other to the heparinized bottle to withdraw the blood from systemic circulation. Electrical activity of the brain, as portrayed by electroencephalography, was used to determine the degree of anesthesia. Physiological parameters were recorded using a 12-channel recorder (Physioscript EE12,® Schwarzer).

After the operative procedure, a period of 30 min was allowed before the hypovolaemic hypotension was induced. The MABP of 55 mm Hg was attained within 15 min and then cautious arterial withdrawals or venous reinfusions by the peristaltic pump were made so that the mean blood pressure was held as constant as possible from 15 to 30 min. Normally, the blood pressure could be held at the specific level within ± 4.0 mm Hg. If large variations occurred or if a substantial part of the blood had to be reinfused to keep the blood pressure constant, the dog was discarded. During the hypovolaemic hypotension, the two experimental conditions were: (1) steady state period of 30 min during air ventilation (PaO_2 100.7 ± 4.9 and $PaCO_2$ 37.7 ± 3.3 mm Hg); at the end of this period, cerebral samples for control determinations were obtained (normoxic normocapnic dogs); (2) steady state period of 15 min during air ventilation plus 15 min of acute hypoxia induced by altering the inspired oxygen concentration with a 5:95 oxygen-nitrogen mixture in the artificial respirator. Under this hypoxic condition PaO_2 ranged between 17 and 19 mm Hg, while $PaCO_2$ ranged between 35 and 40 mm Hg. At the end of this hypoxaemic period, cerebral samples for determinations were obtained (hypoxic normocapnic dogs). During both condition (1) and (2), an intracarotid perfusion (into the superior thyroid arteries) at the rate of 0.1 $ml \cdot min^{-1} \cdot kg^{-1}$ was performed for 30 min (during condition 1) or for 15 min (pretreatment before hypoxia) plus 15 min (treatment during hypoxia) with: (a) saline solution; (b) sodium phenobarbital: 5×10^{-3} M; (c) papaverine hydrochloride: 1×10^{-4} M; (d) (-)eburnamonine phosphate: 5×10^{-4} M; (e) dihydroergocristine methane sulfonate: 1×10^{-5} M.

As concerns the withdrawal of the cerebral samples, at the set time a portion of the motor area of cerebral cortex was removed from the plastic funnel fitted into one side of the skull. The cortical portion of the cerebral

tissue was isolated and immersed in a cold solution of 0.32 M sucrose. The sucrose-washed (0.32 M) and weighed tissue was homogenized in 0.32 M sucrose for 30 sec (precooled Potter-Braun S homogenizer, with a Teflon® pestle rotating at 1000 rev/min) for subsequent synaptosomal preparation. The controlateral portion of the motor area of the brain was frozen *in situ* by liquid nitrogen and removed from the plastic funnel fitted into the other side of the skull. This portion of the brain was cut *in situ* by means of a rotating hollow tube, during liquid nitrogen perfusion. The isolated cerebral material was quickly removed by means of a cooled clamp and then immediately immersed into liquid nitrogen for 10–15 min. The cortical portion of the frozen tissue was isolated and quickly (3–4 sec) powdered in a precooled automatic apparatus (Microdismembrator,® Braun) using frozen 1.23 M perchloric acid. The subsequent steps were carried out in a precooled box at 0–5°C until a neutral perchlorate-free extract was obtained for immediate analyses of metabolites.

For the preparation and incubation of synaptosomes, the diluted (10% w/v) homogenate of the motor area of the cerebral cortex was submitted to fractioning (De Robertis *et al.*, 1962) carried out at 0–4°C, for isolating the crude synaptosomal fraction, obtained as a pellet. The synaptosomal pellet was washed in Krebs-Henseleit-Hepes (144 mM-NaCl; 5 mM-KCl; 5 mM-$NaHCO_3$; 1.3 mM-$MgSO_4$; 1 mM-P_i; 10 mM-Hepes) pH 7.4 buffer and suspended for the subsequent incubation (about 6–8 mg protein/ml) in this medium plus 10 mM-glucose and 2 mM-$CaCl_2$, for 10 min at 24°C. At the set time, the reactions were stopped by the addition of cold perchloric acid, at 3.5% final concentration. The neutralized extract was then used for immediate metabolite analyses.

As concerns the analytical techniques, the cerebral and synaptosomal extracts were utilized for analyses of: ATP (Lamprecht and Trautschold, 1974); ADP and AMP (Jaworek *et al.*, 1974); creatine phosphate (Lamprecht *et al.*, 1974); pyruvate (Czok and Lamprecht, 1974); lactate (Gutmann and Wahlefeld, 1974). The energy charge potential (Atkinson, 1968) was defined in terms of actual concentrations of the adenine nucleotides as: $(|ATP| + 0.5\,|ADP|)/(|ADP| + |ADP| + |AMP|)$. The oxygen consumption of the synaptosomal pellets was measured with a Clark-type oxygen electrode at 24°C in Krebs-Henseleit-Hepes pH 7.4 medium, containing 2 mM-$CaCl_2$ • PaO_2 and $PaCO_2$ were measured using anaerobic samples of blood from the carotid arteries (BMS3-Mk2 Blood Micro System,® Radiometer). Measurements of the cytochrome *c* and *a* content in synaptosomes were performed as described elsewhere (Rafalowska *et al.*, 1980).

For the statistical analysis of the data, the Dunnett's test was applied to the differences in the various instances.

RESULTS

Effect of cerebral hypoxia as a function of the age

As shown in Table 1, under normoxia the metabolite content of the *whole tissue* of the cortical motor area from normocapnic beagle dogs in hypovolaemic hypotension (MABP = 55 ± 4 mm Hg) is practically the same in "young adult," "mature" and "senescent" animals. Only in these last, a slight increase of lactate can be observed, consistent with a slight decrease of creatine phosphate. Table 1 also shows that, in "young adult" dogs, hypoxemia (PaO_2 = 17–19 mm Hg) prolonged for 15 min caused the following changes in the whole tissue of the cerebral cortex motor area: (a) a 50% decrease of creatine phosphate; (b) a 25% decrease of ATP, AMP being up 7.5 times; (c) a 20% decrease of the energy charge potential; (d) a doubling of pyruvate and a tenfold increase of lactate, the lactate-to-pyruvate ratio being thus increased about 4 times. In the tissue as a whole, ageing failed to affect the type and extent of the hypoxic damage, except for a slightly higher increase of AMP and lactate in "mature" and/or "senescent" animals.

Table 2 reveals that, under normoxia conditions, the metabolite content of the *synaptosomal fraction* (isolated from the motor area of the cerebral cortex and incubated for 10 min at 24°C) was virtually the same in the beagle dogs of different ages, except for a slightly increased ADP and/or pyruvate content in "mature" and "senescent" animals. In the synaptosomes isolated from the motor area of "young adults" dogs, hypoxemia caused: (a) a 30% rise in AMP content; (b) a 22% fall in creatine phosphate; (c) 20% increase of pyruvate; (d) a 50% increase of lactate, the lactate-to-pyruvate ratio being increased by about 25%. The effects of hypoxia on the synaptosomal fraction were markedly affected by ageing, since "mature" and "senescent" dogs exhibited: (a) a higher

TABLE 1. MOTOR AREA OF CEREBRAL CORTEX FROM NORMOCAPNIC BEAGLE DOGS IN HYPOVOLAEMIC HYPOTENSION (MABP = 55 ± 4 mmHg). METABOLITES CONTENT EVALUATED IN FROZEN CORTICAL TISSUES (μmoles · g^{-1}). BEHAVIOR IN NORMOXIA AND AFTER 15 MIN OF HYPOXAEMIA (PaO_2 = 17–19 mmHg)

	"Young adult" dogs		"Mature" dogs		"Senescent" dogs	
Metabolite or ratio	Normoxia (20)	Hypoxia (16)	Normoxia (6)	Hypoxia (6)	Normoxia (3)	Hypoxia (3)
ATP	2.38 ± 0.04	1.79 ± 0.12*	2.49 ± 0.05	1.64 ± 0.06*	2.29 ± 0.09	1.55 ± 0.03*
ADP	0.41 ± 0.02	0.59 ± 0.02*	0.45 ± 0.03	0.61 ± 0.05*	0.53 ± 0.09	0.65 ± 0.03*
AMP	0.07 ± 0.01	0.53 ± 0.10*	0.07 ± 0.01	0.67 ± 0.03*▲	0.09 ± 0.01	0.72 ± 0.05*▲
ΣAdenine nucleotides	2.86 ± 0.01	2.91 ± 0.03	3.01 ± 0.06	2.51 ± 0.08*	2.91 ± 0.09	2.92 ± 0.04
Energy charge	0.903 ± 0.003	0.715 ± 0.032*	0.902 ± 0.001	0.666 ± 0.001*	0.878 ± 0.001	0.643 ± 0.001*
Creatine phosphate	4.62 ± 0.11	2.11 ± 0.16*	4.09 ± 0.09	2.51 ± 0.08*	3.76 ± 0.13 ▲	2.07 ± 0.08*
Pyruvate	0.111 ± 0.004	0.269 ± 0.024*	0.110 ± 0.006	0.283 ± 0.008*	0.110 ± 0.001	0.303 ± 0.001*
Lactate	2.13 ± 0.16	20.59 ± 1.36*	2.33 ± 0.08	23.47 ± 2.21*	2.59 ± 0.06 ▲	26.30 ± 1.14*▲
Lactate/pyruvate	19.09 ± 1.12	77.06 ± 3.05*	21.26 ± 0.93	83.66 ± 5.43*	23.74 ± 2.25	87.01 ± 5.50*

Results are expressed as mean values ± S.E. for the number of experiments indicated in parentheses.
*Differs from normoxic animals ($p < 0.05$).
▲Differs from "young adult" animals ($p < 0.05$).

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TABLE 2. MOTOR AREA OF CEREBRAL CORTEX FROM NORMOCAPNIC BEAGLE DOGS IN HYPOVOLAEMIC HYPOTENSION (MABP = 55 ± 4 mmHg). METABOLITES CONTENT EVALUATED IN SYNAPTOSOMAL PELLETS AFTER FURTHER INCUBATION FOR 10 MIN AT 24° C (nmoles • mg protein^{-1}). BEHAVIOR IN NORMOXIA AND AFTER 15 MIN OF HYPOXAEMIA (PaO_2 = 17–19 mmHg)

	"Young adult" dogs		"Mature" dogs		"Senescent" dogs	
Metabolite or ratio	Normoxia (20)	Hypoxia (16)	Normoxia (6)	Hypoxia (6)	Normoxia (3)	Hypoxia (3)
ATP	5.45 ± 0.10	5.06 ± 0.20	5.03 ± 0.16	3.92 ± 0.10*▲	4.87 ± 0.15	3.23 ± 0.19*▲
ADP	1.74 ± 0.05	1.82 ± 0.10	2.02 ± 0.05	2.33 ± 0.15 ▲	2.17 ± 0.19 ▲	2.51 ± 0.21 ▲
AMP	0.43 ± 0.02	0.56 ± 0.02*	0.49 ± 0.04	1.44 ± 0.16*▲	0.50 ± 0.04	1.83 ± 0.05*▲
ΣAdennine nucleotides	7.62 ± 0.07	7.44 ± 0.12	7.55 ± 0.20	7.69 ± 0.16	7.54 ± 0.08	7.57 ± 0.38
Energy charge	0.829 ± 0.003	0.801 ± 0.009	0.800 ± 0.001	0.661 ± 0.001*▲	0.790 ± 0.001	0.591 ± 0.001*▲
Creatine phosphate	5.42 ± 0.10	4.20 ± 0.28*	4.97 ± 0.11	3.20 ± 0.14*▲	4.61 ± 0.19 ▲	3.00 ± 0.07*▲
Pyruvate	5.82 ± 0.15	7.03 ± 0.21*	7.02 ± 0.26 ▲	7.94 ± 0.62	7.34 ± 0.41 ▲	7.90 ± 0.17
Lactate	22.08 ± 0.56	33.06 ± 1.04*	25.80 ± 2.19	36.64 ± 1.10*	24.82 ± 1.30	37.24 ± 1.98*
Lactate/pyruvate	3.83 ± 0.12	4.76 ± 0.20*	3.68 ± 0.29	4.76 ± 0.39*	3.39 ± 0.13	4.73 ± 0.32*

Results are expressed as mean values ± S.E. for the number of experiments indicated in parentheses.

* Differs from normoxic animals ($p < 0.05$).

▲ Differs from "young adult" animals ($p < 0.05$).

increase of AMP (190% and 265%, respectively); (b) a decrease of ATP content (22% and 34%, respectively); (c) a decrease of the energy charge potential (17% and 25%, respectively); (d) a more evident decrease of the creatine phosphate content (about 35%). On the contrary, the hypoxic changes in the content of the representative participants of the glycolytic pathway were unaffected by ageing, the increase of the lactate-to-pyruvate ratio being of the same order of magnitude in "mature" and "senescent" dogs as in "young adult" ones.

Table 3 shows that the oxygen consumption rate in the synaptosomal fraction from the motor area of the hypoxic beagle dogs failed to be significantly affected by ageing. On the contrary, the content of cytochromes *c* and *a* was significantly reduced in the "senescent" dogs. This demonstrates that the turnover rate for cytochromes *c* and *a* is higher in the synaptosomes from old brains.

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Effects on synaptosomes of the pharmacological treatment in normocapnic hypoxic "young adult" and "mature" animals

The evaluation of the biochemical parameters ATP, ADP, AMP, creatine phosphate, lactate and pyruvate of the synaptosomal preparations from the cerebral cortex motor area of beagle dogs (Table 2) proved to be a much better index of the effect of cerebral hypoxia than the evaluation of the same effect carried out on the cortical motor area tissue as a whole (Table 1). Indeed, in the synaptosomal fraction (Table 2) a high sensibilization to the hypoxic damage can be observed in "mature" and "senescent" dogs as compared with "young adult" ones. Though contributing to the understanding of hypoxia physiopathology, the evaluation of oxygen consumption rate and cytochromes level in the synaptosomal pellets (Table 3) proved to be an index of hypoxic damage sensibilization only in the "senescent" dogs. Now, the study of the action of a drug implies the availability of an adequate number of homogeneous animals. Such number, multiplied by the 4

TABLE 3. OXYGEN CONSUMPTION RATES AND CYTOCHROME LEVELS IN SYNAPTOSOMAL PELLETS FROM THE MOTOR AREA OF THE CEREBRAL CORTEX OF NORMOXIC AND HYPOXIC BEAGLE DOGS IN HYPOVOLEMIC HYPOTENSION (MABP = 55 ± 4 mmHg) AS A FUNCTION OF AGE

		Hypoxic animals		
Biochemical parameters	"Young-adult" normoxic animals (10)	"Young-adult" (10)	"Mature" (6)	"Senescent" (3)
Oxygen consumption rate nmoles • min^{-1} • (mg protein)$^{-1}$	2.91 ± 0.23*	1.65 ± 0.36	1.55 ± 0.21	1.49 ± 0.20
CYTOCHROME *c* nmoles • (mg protein)$^{-1}$	0.13 ± 0.02*	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.01*
CYTOCHROME *a* nmoles • (mg protein)$^{-1}$	0.10 ± 0.01*	0.07 ± 0.01	0.06 ± 0.00	0.04 ± 0.00*

Results are expressed as mean values ± S.E. for the number of experiments indicated in parentheses.
*Differs from hypoxic young-adult animals ($p < 0.05$): Dunnett's test.

treatments scheduled, necessarily limited this investigation to "young adult" (1.5 years of age) and "mature" (6.5 years of age) dogs. Under these circumstances, the synaptosomal parameters reported in Table 2 proved the most suitable to point out the effect of a drug, if any, on the hypoxic damage induced in "mature" animals, in relation to a similar damage induced in "young adult" ones.

Table 4 illustrates the effect of acute hypoxia (15 min) on the synaptosomal fraction from the motor area of the cerebral cortex of untreated and treated "*young adult*" beagle dogs. Under hypoxia conditions, the intracarotid perfusion of 0.1 ml • min^{-1} • kg^{-1} for 15 min (before hypoxia) plus 15 min (during hypoxia) of *papaverine* (1×10^{-4} M) did not affect the content of the metabolites tested, except for a slight pyruvate increase. On the contrary, under acute hypoxia, the intracarotid perfusion with *phenobarbital* (5×10^{-3} M), as compared to hypoxic untreated dogs, caused: (a) an increase of ATP (122%), total adenine nucleotides content (77%), creatine phosphate (90%) and energy charge potential (14%) to values much higher even than those of normoxic untreated animals; (b) a decrease of AMP (46%) to values lower even than those of normoxic untreated animals; (c) a decrease of pyruvate (62%) and lactate (57%) to values lower even than those of normoxic untreated animals. In hypoxic "young adult" beagle dogs, the intracarotid perfusion with (-)*eburnamonine* (5×10^{-4} M) caused, as compared with hypoxic untreated dogs: (a) an increase of ATP (58%), total adenine nucleotides content (37%), creatine phosphate (43%) and energy charge potential (9%) to values higher even than those of normoxic untreated dogs; (b) a decrease of AMP content (32%); (c) no change in the lactate and pyruvate content. Finally, under hypoxia, the intracarotid perfusion with *dihydroergocristine* (1×10^{-5} M), as compared to hypoxic untreated dogs, caused: (a) an increase of ATP (88%), total adenine nucleotides content (58%) and energy charge potential (10%) to values higher even than those of normoxic untreated dogs; (b) no change in the AMP and creatine phosphate content; (c) a moderate increase of the lactate content (21%) and of the lactate-to-pyruvate ratio (24%).

Table 5 shows the effect of acute hypoxia (15 min) of the synaptosomal fraction from the motor area of the cerebral cortex of untreated and treated "*mature*" beagle dogs. Under hypoxia, the intracarotid perfusion of 0.1 ml • min^{-1} • kg^{-1} for 15 min (before hypoxia) plus 15 min (during hypoxia) of *papaverine* (1×10^{-4} M) had no effect on the content of the metabolites tested. On the contrary, under acute hypoxia, the intracarotid perfusion with *phenobarbital* (5×10^{-3} M), as compared to hypoxic untreated dogs, caused: (a) an increase of ATP (120%), total adenine nucleotides content (51%), creatine phosphate (62%) and energy charge potential (27%) to values higher even than those of normoxic untreated dogs; (b) a decrease of AMP (58%); (c) a decrease of pyruvate (42%) and lactate (46%) to values lower even than those of normoxic untreated dogs. In hypoxic "mature" beagle dogs, the intracatorid perfusion with (-)*eburnamonine* (5×10^{-4} M), as compared to hypoxic untreated dogs, caused: (a) an increase of ATP (41%), total adenine nucleotides content (22%) and energy charge potential (9%); (b) no change in AMP and creatine phosphate content; (c) no change in the pyruvate and lactate content. Finally, under hypoxia, the intracarotid perfusion with *dihydroergocristine* (1×10^{-5} M), as compared to hypoxic untreated dogs, caused: (a) an increase of ATP (45%), total adenine nucleotides content (22%) and energy charge potential (10%); (b) no change in the creatine phosphate and AMP content; (c) a moderate increase of the lactate content (21%) and of the lactate-to-pyruvate ratio (25%).

DISCUSSION

The data reported refer to the motor area of the cerebral cortex and therefore other important areas of the brain might undergo different biochemical adjustments, because of the well-known heterogeneity of the organ. On the other hand, this paper does not intend to tackle the problem of describing or predicting therapeutic measures with regard to ageing in humans. In fact, drugs are utilized as tools able or unable to affect the cerebral reactivity in a biological model of acute hypoxia. In any case, the present data can not be acritically transferred to the field of human physiopathology.

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In the untreated beagle dogs, the experimental model shows in the two opposite cortical motor areas of the same animal, two clearly distinct biochemical situations. The first (surface freezing technique) describes the actual energy state of the cortical motor area following acute hypoxia. The second (incubation technique) describes the potential damage the synaptosomes of the controlateral cortical motor area "remember" as a consequence of acute hypoxia. Therefore, the first observation yields a flash of the overall energy level of the motor cortical tissue after hypoxia. The second observation provides an evaluation of the specific function of synaptosomes when, after hypoxia *in vivo*, they are put under an optimal incubation condition *in vitro*. At any rate, the choice of synaptosomes as the subcellular fraction to be tested, stemmed from the well-known role played by plasma membrane in these nerve ending particles.

An examination of the data of Table 1 points out that the basal biochemical situation of the cerebral motor area tissue as a whole is similar in "young adult," "mature" and "senescent" animals. Furthermore, it must be stressed that, in these dogs of different ages, the hypoxia is responsible for similar effects on the overall actual energy state of the cortical tissue tested as a whole. Therefore, ageing does not seem to affect the type and extent of the overall biochemical derangement caused by the hypoxia. On the contrary, a quite different situation can be observed when (Table 2) the effects of hypoxia at subcellular level are studied by evaluating the changes that the synaptosomes "remember" and which can not be reversed during the *in vitro* incubation of these nerve ending particles. In this instance, ageing slows down the functional recovery of the incubated synaptosomes. Indeed (Table 2), their biochemical derangement is increasingly evident in "young adult," "mature" and "senescent" dogs, in this order. Therefore, ageing does not so much affect the cerebral changes that occur in the overall energetic state during the hypoxic assault *in vivo*, but rather those that the subcellular components (such as the synaptosomes) remember and tend to reverse during the recovery period of incubation *in vitro*.

On the other hand, it can be seen in Table 3 that although oxygen consumption is the same in the synaptosomes from the motor area of hypoxic "young adult," "mature" and "senescent" dogs, the cytochromes *c* and *a* content is lower in the latter preparation. This points to higher turnover rates for cytochromes in the synaptosomes from the motor area of older animals. The most likely explanations for the lower post-hypoxic content of cytochromes *c* and *a* in old brains are: (1) age-dependent changes in the protein-turnover machinery, inducing changes in the rate of synthesis and/or degradation of cytochromes; (2) age-dependent changes in the morphology of synaptosomes caused by the fact that the releasing process (which leads to the formation of synaptosomes) yields vesicles that include different numbers of mitochondria depending on the different initial state of the "young" or "old" cortical tissue.

As to the pharmacological effect of phenobarbital, an evident protective action on the incubated synaptosomal fraction can be observed after hypoxia both in "young adult"

(Table 4) and in "mature" animals (Table 5). This in the absence of marked action differences as a function of ageing. The protective effect of the barbiturate at synaptosomal level can be related to some specific pharmacodynamic characteristics, such as: (1) lowering of the body or brain temperature; (2) inhibition of the sympathetic discharge that occurs during the withdrawal of the cerebral samples; (3) stabilization of the plasma membrane of the nerve ending particles; (4) inhibition of the activity of some enzymes related to energy transduction (e.g., phosphofructokinase, malate dehydrogenase); (5) activation of the GABAergic system. These pharmacological properties account for the cerebral sparing action pointed out by the effect of phenobarbital on what the synaptosomes "remember" of the hypoxic damage, and which is reverted during incubation (Tables 4 and 5). In clinical experience, it is of great interest that barbiturates may facilitate neurological restitution after a cerebral damage, such as ischemia or hypoxia. Unfortunately, the main drawback that restricts their use in clinical practice is that it is impossible to avoid anesthesia. On the other hand, the anestetic effect can be regarded as at least partially independent of the pharmacological mechanisms described above. For example, with phenobarbital (Benzi *et al.*, 1979b) or althesin (Benzi *et al.*, 1980c) the anesthesia is no longer present at times when an interference with several cerebral enzymatic activities related to energy transduction or redox state can be demonstrated. It is therefore reasonable to investigate at cerebral level the effect of substances devoid of anesthetic action, which however exhibit pharmacological characteristics at least partially similar to those of barbiturates.

In normocapnic hypoxic animals, the intracarotid perfusion with papaverine fails to modify, at synaptosomal level, the biochemical parameters examined. On the contrary, (-)eburnamonine increases the synaptosomal phosphorylation state (energy charge, total adenine nucleotides content, and creatine phosphate storage), being ineffective on the glycolytic metabolites tested. The effect of the drug is however more marked in "young adult" dogs (Table 4) as compared to "mature" ones (Table 5). In the latter group, creatine phosphate is no longer increased, while ATP and total adenine nucleotides content exhibit an increase which is, respectively, 30% and 40% lower than that found in the synaptosomal preparations from the motor area of "young adult" brains. On the other hand, the (-)eburnamonine-induced rise in the energy charge potential is similar in "young adult" and "mature" animals. The intracarotid perfusion of dihydroergocristine in normocapnic hypoxic beagle dogs increases the synaptosomal phosphorylation state (energy charge and total adenine nucleotides content), interfering also with the metabolites of anaerobic glycolysis. Also with dihydroergocristine, the pharmacological effect is more marked in "young adult" dogs (Table 4) than in "mature" ones (Table 5). In the latter group the increase of ATP and total adenine nucleotides content is, respectively, 50% and 60% lower than the one observed in the synaptosomal preparations from the motor area of "young adult" beagle dogs. In any case, the increase induced by dihydroergocristine on the energy charge potential is similar in the synaptosomal preparations from the cortical motor area of both "young adult" and "mature" dogs.

The true nature of the modifications which underlie the hypoxic damage as a function of age is unknown, though probably involving changes in the enzymatic activities, modifications of the levels of intracellular ions, alterations of synaptic transmission, or derangements in the structure of membranes. In any case, ageing does not magnify the damages caused by the hypoxic assault on the overall energetic state (Table 1), while providing a tissular condition owing to which the hypoxic damage is more difficult to be

TABLE 4. "YOUNG ADULT" DOGS—MOTOR AREA OF CEREBRAL CORTEX FROM NORMOCAPNIC "YOUNG ADULT" BEAGLE DOGS IN HYPOVOLAEMIC HYPOTENSION (MABP = 55 ± 4 mm Hg). METABOLITES CONTENT (nmoles • mg protein^{-1}) IN SYNAPTOSOMAL PELLETS (AFTER INCUBATION FOR 10 MIN AT 24°C) OBTAINED FROM UNTREATED OR PRETREATED (FOR 15 MIN) AND TREATED (FOR 15 MIN) HYPOXIC ANIMALS (PaO_2 = 17–19 mm Hg, FOR 15 MIN)

			Hypoxic animals pretreated and treated with intracarotid perfusion (0.1 ml • min^{-1} • kg^{-1} for 15 + 15 min)			
Metabolite or ratio	Normoxic untreated animals (20)	Hypoxic untreated animals (16)	Phenobarbital 5×10^{-3}M (6)	Papaverine 1×10^{-4}M (5)	(-)Eburnamonine 5×10^{-4}M (5)	Dihydroergocristine 1×10^{-5}M (6)
ATP	5.45 ± 0.10	5.06 ± 0.20	11.23 ± 0.46*	4.73 ± 0.21	7.98 ± 0.51*	9.52 ± 0.53*
ADP	1.74 ± 0.05	1.82 ± 0.10	1.66 ± 0.15	1.97 ± 0.21	1.84 ± 0.20	1.71 ± 0.11
AMP	0.43 ± 0.02*	0.56 ± 0.02	0.30 ± 0.03*	0.63 ± 0.06	0.38 ± 0.03*	0.51 ± 0.04
ΣAdenine nucleotides	7.62 ± 0.07	7.44 ± 0.12	13.20 ± 0.32*	7.33 ± 0.26	10.20 ± 0.56*	11.73 ± 0.43*
Energy charge	0.829 ± 0.003	0.801 ± 0.009	0.913 ± 0.008*	0.779 ± 0.005	0.872 ± 0.011*	0.882 ± 0.011*
Creatine phosphate	5.42 ± 0.10*	4.20 ± 0.28	7.96 ± 0.34*	3.83 ± 0.25	6.02 ± 0.31*	3.83 ± 0.25
Pyruvate	5.82 ± 0.15*	7.03 ± 0.21	2.65 ± 0.20*	8.21 ± 0.42*	6.63 ± 0.32	6.83 ± 0.20
Lactate	22.08 ± 0.56*	33.06 ± 1.04	14.36 ± 0.70*	34.63 ± 1.69	34.81 ± 1.23	39.96 ± 1.32*
Lactate/pyruvate	3.83 ± 0.12*	4.76 ± 0.20	5.54 ± 0.44	4.26 ± 0.29	5.28 ± 0.24	5.90 ± 0.37*

Results are expressed as mean values ± S.E. for the number of experiments indicated in parentheses.
*Differs from hypoxic untreated animals ($p < 0.05$): Dunnett's test.

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TABLE 5. "MATURE" DOGS – MOTOR AREA OF CEREBRAL CORTEX FROM NORMOCAPNIC "MATURE" BEAGLE DOGS IN HYPOVOLAEMIC HYPOTENSION (MABP = 55 ± 4 mm Hg). METABOLITES CONTENT (nmoles • mg protein^{-1}) IN SYNAPTOSOMAL PELLETS (AFTER INCUBATION FOR 10 MIN AT 24° C) OBTAINED FROM UNTREATED OR PRETREATED (FOR 15 MIN) AND TREATED (FOR 15 MIN) HYPOXIC ANIMALS (PaO_2 = 17–19 mm Hg, for 15 min).

			Hypoxic animals pretreated and treated with intracarotid perfusion (0.1 ml • min^{-1} • kg^{-1} for 15 + 15 min)			
Metabolite or ratio	Normoxic untreated animals (6)	Hypoxic untreated animals (6)	Phenobarbital 5×10^{-3}M (3)	Papaverine 1×10^{-4}M (3)	(-)Eburnamonine 5×10^{-4}M (3)	Dihydroergocristine 1×10^{-5}M (3)
ATP	5.03 ± 0.16*	3.92 ± 0.10	8.62 ± 1.10*	3.82 ± 0.26	5.54 ± 0.21*	5.70 ± 0.26*
ADP	2.02 ± 0.05	2.33 ± 0.15	2.41 ± 0.39	2.45 ± 0.19	2.52 ± 0.18	2.24 ± 0.20
AMP	0.49 ± 0.04*	1.44 ± 0.16	0.60 ± 0.02*	1.51 ± 0.14	1.32 ± 0.05	1.43 ± 0.11
ΣAdenine nucleotide	7.55 ± 0.20	7.69 ± 0.16	11.63 ± 0.77*	7.78 ± 0.03	9.38 ± 0.33*	9.37 ± 0.35*
Energy charge	0.800 ± 0.001*	0.661 ± 0.001	0.842 ± 0.026*	0.648 ± 0.026	0.724 ± 0.007*	0.728 ± 0.017*
Creatine phosphate	4.97 ± 0.11*	3.20 ± 0.14	5.18 ± 0.76*	3.45 ± 0.24	3.27 ± 0.22	3.04 ± 0.10
Pyruvate	7.02 ± 0.26	7.94 ± 0.62	4.61 ± 0.22*	8.26 ± 0.45	6.76 ± 0.28	7.43 ± 0.21
Lactate	25.80 ± 2.19*	36.64 ± 1.10	19.76 ± 2.36*	38.32 ± 1.76	39.24 ± 1.72	44.21 ± 1.63*
Lactate/pyruvate	3.68 ± 0.29*	4.76 ± 0.39	4.36 ± 0.71	4.65 ± 0.26	5.81 ± 0.04*	5.97 ± 0.35*

Results are expressed as mean values ± S.E. for the number of experiments indicated in parentheses.
*Differs from hypoxic untreated animals ($p < 0.05$): Dunnett's test.

repaired during the incubation of synaptosomes *in vitro* (Tables 2 and 3). The markedly favorable effect of phenobarbital in both "young adult" and "mature" dogs suggests that its spectrum of pharmacodynamic action is sufficiently broad to exert a quite effective sparing action (Tables 4 and 5), even if accompanied with anesthesia. The more limited effects of (-)eburnamonine and dihydroergocristine, which decrease with ageing, suggests a clearly narrower spectrum of interference with the assumed pathogenetic mechanisms, such as the morphological (i.e., structure of membranes), physiological (i.e., synaptic transmission), biochemical (i.e., enzymatic activities related to energy transduction) or biophysical (i.e., levels of intracellular ions) factors.

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SUMMARY

The synaptosomal fraction obtained from the motor area of the cerebral cortex of normocapnic, normoxic or hypoxic "young adult," "mature" and "senescent" beagle dogs is incubated and analyzed for: ATP, ADP, AMP, creatine phosphate, pyruvate and lactate. The data are compared with those obtained from the whole controlateral cortical motor area, by the surface freezing technique. After hypoxic hypoxia (15 min; PaO_2 = 17–19 mm Hg), the metabolite contents and ratios are differently affected by ageing when the evaluations are performed in the incubated synaptosomal preparation or in the controlateral whole cerebral tissue. In fact, ageing does not affect so much the cerebral changes that occur in the overall energetic state during the hypoxic assault *in vivo*, but rather those that the synaptosomes remember and tend to reverse during the subsequent incubation *in vitro*.

The protective action of several drugs on the synaptosomal phosphorylation state is tested. Phenobarbital shows a quite broad, age-independent spectrum of action. (-)Eburnamonine and dihydroergocristine exhibits a more limited, age-dependent effectiveness, but are devoid of anesthetic action. Papaverine proves unable to affect the tested biochemical parameters.

Acknowledgements—The authors wish to thank Dr. M.L. Riva for assistance in the preparation of the manuscript, and Mrs. G. Garlaschi, Mr. L. Maggi and Mr. G. Arioli for technical assistance.

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Experimental Gerontology, Vol. 17, pp. 33–40, 1982
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AGE RELATED CHANGES IN MORPHOLOGY OF THE THYMUS OF THE FISH, *ORYZIAS LATIPES*

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(*Received* 12 *March* 1981)

INTRODUCTION

AGE RELATED involution of the thymus in mammals is well known, and it has been suggested to be an important role of the organ in aging processes in relation to its immunological function. As to the thymus in teleost fish, however, no systematic observations of thymus size throughout life have been reported. We have already published the standard life table of the fish, *Oryzias latipes* (Egami and Etoh, 1969; Egami, 1971), and in the present paper the age related change in the size and histology of this species will be described in order to provide a better understanding of aging mechanisms in the fish.

MATERIALS AND METHODS

The materials were the orange red variety of the medaka, *Oryzias latipes* which had been kept for 0–5 years in large plastic containers put on the roof of our building under natural conditions and fed Tetramin (Tetra Werke, West Germany) almost every day. The maximum life span was about 5.5 years in the materials. Healthy looking three males and three females of 1-, 2-, 3-, 4-year-old groups were picked up from the containers and fixed in Bouin's fluid. In the 5-year-old group the number of survivors was small and only two males and two females were used. All of these fish were fixed in May. Also, six young fish of 3 months old were examined in July. After the body length and weight examination, the thymus regions were embedded in paraffin, sectioned seriously 5μ thick, and stained with haemalum and eosin. After histological observation, the border of all thymus sections was drawn and the area measured with planimeter and mean thymus volume was calculated as reported in our previous paper (Ghoneum et al., 1979a, 1979b).

OBSERVATIONS

1. General description of the thymus development

Embryonic development of the thymus of normal fish has already been reported (Ghoneum *et al.*, 1981). After hatching, the thymus increased in size and differentiation

The results of this study were briefly read at the Symposium of "Comparative Biology of Aging" in the 1st Asia/Oceania Congress of Gerontology, Melbourne, December 1980

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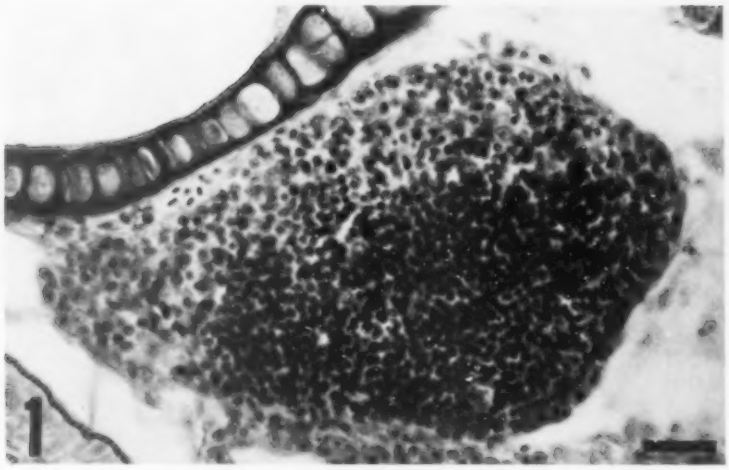

FIG. 1. Thymus examined at 20 days after hatching. Note the differentiation of the thymus into the dark zone (densely packed with thymocytes) and the light zone. The bar represents 20 μm.

of dark and light zones of the thymus became distinct at 20 days after hatching (Fig. 1). In 3-month-old young, the thymus developed and the anterior and posterior lobes of the organ, each of the lobes consisting of two zones, were clearly differentiated (Fig. 2). Afterwards, general morphology of the thymus did not change throughout the life of the fish. Very distinct seasonal change in the volume of the thymus appeared in this fish (Ghoneum, in press); they increased during the breeding season, extending from April to September under the present conditions. Sampling of the fish of the present experiment, therefore, was carried out in May except for the fish of 3 months old.

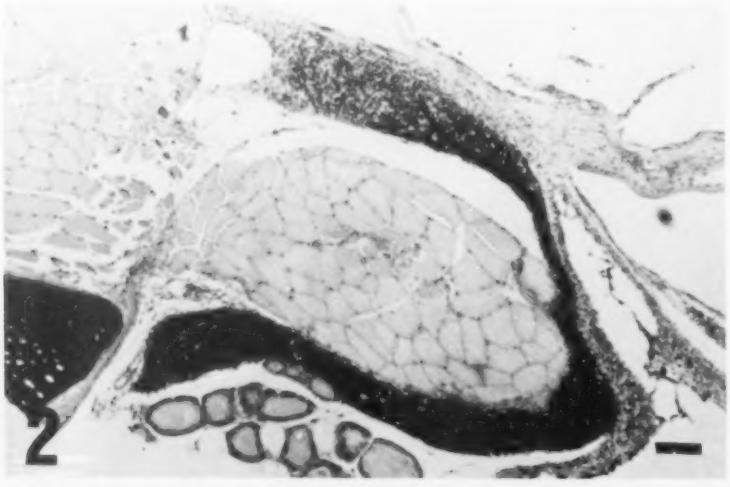

FIG. 2. The thymus of 3-month-old fish; it consists of two lobes, anterior lobe and posterior one, partially divided by muscle band. The bar represents 50 μm.

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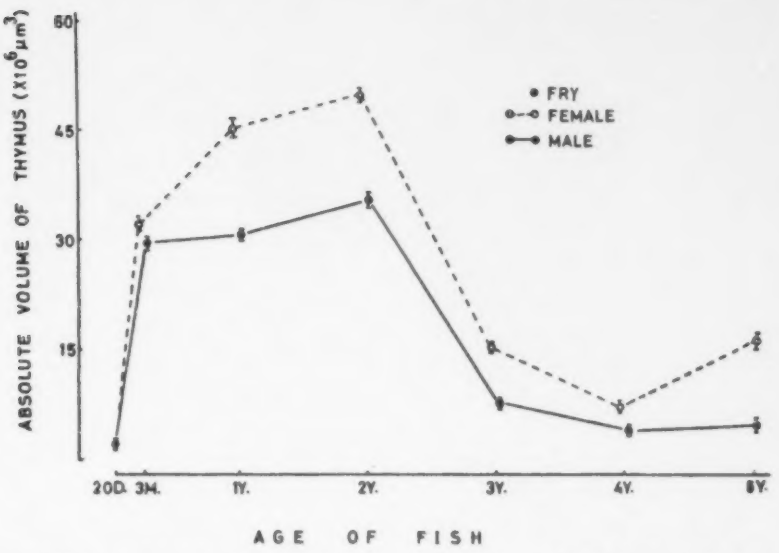

FIG. 3. Age-related change in absolute volume of the thymus in the fish, *Oryzias latipes*.

2. *Thymus volume of fish at different ages*

Means of the thymus volumes of male and female fish at different ages are given in Fig. 3. From this figure it is found that thymus volume increased very rapidly during 3 months of the early development, however it increased very slowly during two years. However, the thymus decreased in size in the old stage. The thymus of the female in 2 and 3-year-old is significantly larger than that of the male. It is worth mentioning that the fish of 3- and 4-year-old groups could lay eggs. However, fertility of fish of the 5-year-old group was very low.

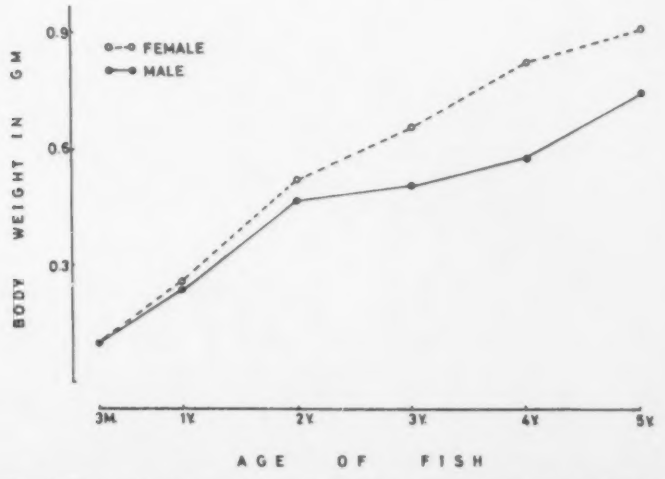

FIG. 4. General body growth in male and female of the fish, *Oryzias latipes*.

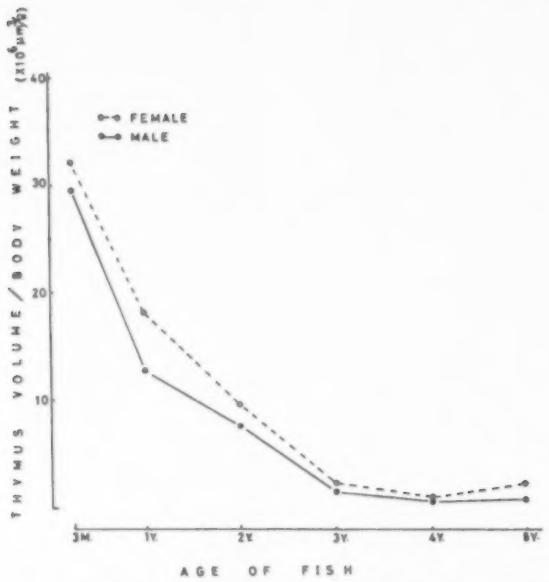

FIG. 5. Relative volume of the thymus in the fish, *Oryzias latipes*.

In this fish, both body length and weight continued to increase until senescence (Fig. 4) and the relative volume of the thymus was calculated by the ratio of the thymus volume to body weight (Fig. 5). From this figure involution of the organ is clearly demonstrated from 3 months after hatching in both sexes.

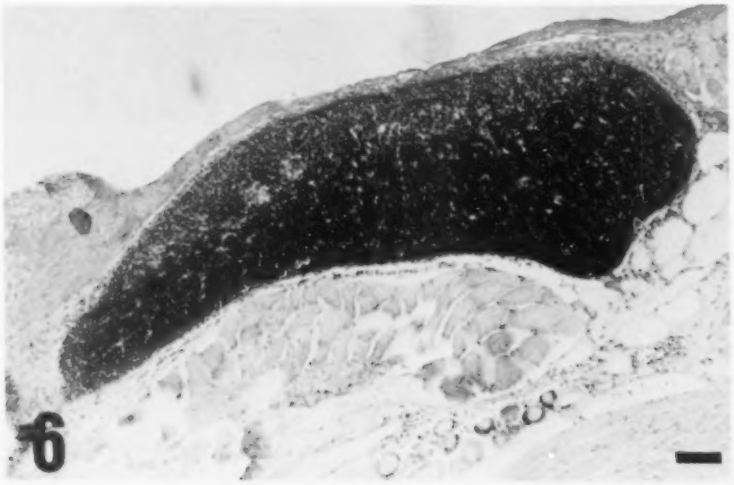

FIG. 6. The anterior lobe of the thymus of 2-year-old fish. Note differentiation into dark and light zones. The bar represents 50 μm.

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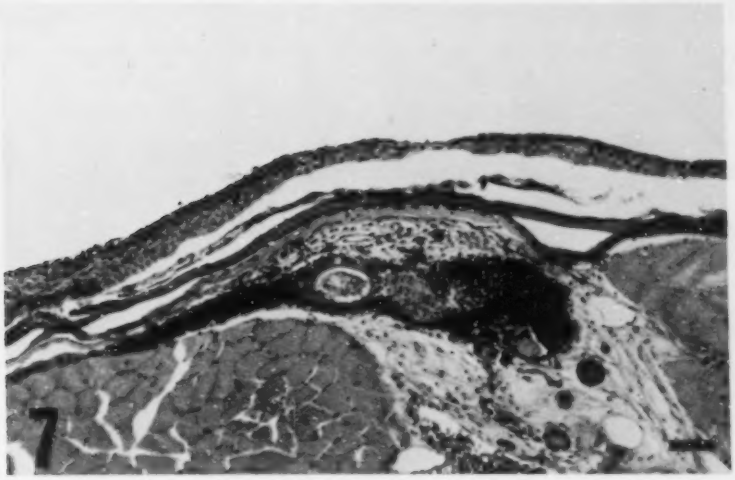

FIG. 7. A cross-section of a 3-year-old thymus, showing reduction in volume, increase in the amount of connective tissue around and within the organ. Note cystic space and lost demarcation between dark and light zones. The bar represents 50 μm.

3. Histology of fish at different age

In the fry at 20 days after hatching, the thymus contained densely packed lymphocytes. At 3 months, the thymus was partially divided by muscle into two lobes, each lobe consisting of a dark zone which was occupied by mainly lymphocytes, and a light zone which contained lympocytes and reticular cells. Small numbers of mitotic figures were found. In fish of 1 and 2 years old, the thymus during breeding seasons was histologically almost

FIG. 8. Part of the left thymus of 3-year-old fish, showing emmigration of thymocytes into adjacent connective tissues. The bar represents 50 μm.

similar to that in fish of 3 months old. In fish of 3 years old, differentiation of the zone became unclear and the density of lympocytes decreased and reticular cells and collagenous fibers in parenchyma increased and some cystic space was filled with mucus (Figs. 6, 7 and 8). In 4-year-old fish, the degenerating characteristics were more advanced and the amount of connective tissues and cysts in the organ increased. In 5-year-old fish, the organ became thin in shape and the total number of thymocytes was very small (Figs. 9, 10 and 11).

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DISCUSSION

Usefulness of fish in aging research has been well pointed out by Woodhead (1978). Several authors reported age involution of the thymus in fish. Deanesly (1927) published complete disappearance of the thymus in female trout, the time of its disappearance varying with the individual. Lele (1933) reported that in the plaice, the thymus continues to grow up to the first spawning—which occurs during its third year—and then diminishes steadily and slowly until the fish become 7 years old, when actual thymic involution begins. Correlation between age involution in the thymus with the sexual maturity of fish was observed by Hafter (1952) in *Astyanax* which showed the beginning of thymic involution at the time of sexual maturity, which occurred at about 26 weeks of age, while Von Hagen (1936) said that in *Anguilla volgaris* of 15–20 cm in length, these fish are presumably not sexually mature. Deanesly (1927) also stated that there was no evidence that involution of the thymus of fish, *Salmo fario* was associated with the time of sexual maturity. Deanesly (1927) thought that a mass emmigration of small lymphocytes from the organ was a factor in its involution. The histological examinations of the thymus of the fish, *Oryzias latipes* indicated that emmigration of lymphocytes took place at all ages.

FIG. 9. The thymus of 5 year-old fish, showing cyst occupying most of the organ. The bar represents 100 μm.

FIG. 10. Higher magnification of a part of Fig. 9, showing the enlargement of the cystic spaces filled with mucus and debris derived from degenerated cells. The bar represents 100 μm.

Present observation clearly shows that the thymus of *Oryzias latipes* developed very early and relative size decreased logarithmically after first maturation of the fish. During senescent stage the organ became very thin. The age-related change is similar to mammalian species. These similarities between mammals and fish seem to suggest the basic

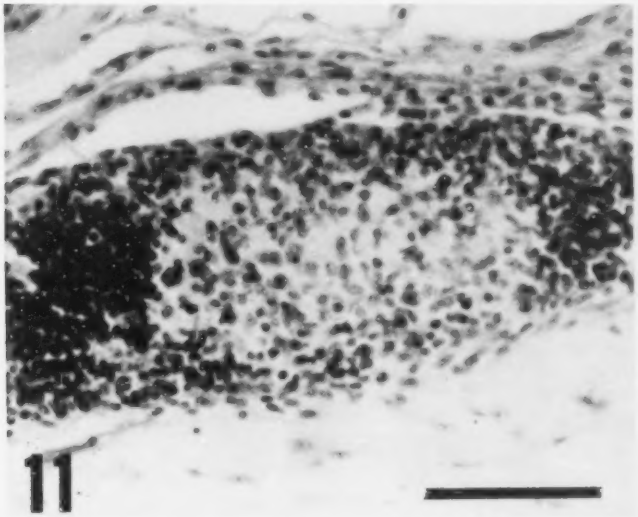

FIG. 11. The thymus of 5-year-old fish, showing increase in connective tissue proliferation that occupied most of the organ. The bar represents 100 μm.

similarity in the important role of the thymus in the aging process of the vertebrate in animals.

SUMMARY

The thymus of the teleost fish *Oryzias latipes* is a paired structure found at the dorso-posterior part of the gill chamber. In 3-month-old fish, the thymus shows a great development. The thymus displays atrophy during aging, and the thymus involution continues until 5 years of age. Male thymus shows heavier involution than female thymus of the same age. Emigration of thymus cells takes place at all ages but increases with age.

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Acknowledgements—This study was supported in part by a Grant-in Aid for Fundamental Scientific Research from the Ministry of Education, Science and Culture to N. E. We wish to express our thanks to Drs. K. Ijiri and A. Kikuta for their help during the course of this work.

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Experimental Gerontology, Vol. 17, pp. 41–48, 1982
Printed in the USA.

0531-5565/82/010041-08$03.00/0
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CHANGES IN HEPATIC MICROSOMAL MEMBRANE FLUIDITY WITH AGE

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(*Received* 12 *March* 1981)

INTRODUCTION

THE HEPATIC MIXED function oxidase system is responsible for the metabolism of a wide variety of compounds including carcinogens, fatty acids, and xenobiotics. The metabolism of many of these compounds by the whole animal changes with age. In the rat, the *in vivo* rate of metabolism of several compounds is lower in the old compared to the young (Baird *et al.*, 1971; Baird *et al.*, 1975). In *in vitro* studies, hepatic microsomes prepared from old rats have decreased levels of ethyl-morphine • N-demethylase, benzphetamine • N-demethylase, and benzo(a)-pyrene hydroxylase activity compared to microsomes from young rats (Birnbaum and Baird, 1978a). However, in these same animals the level of cytochrome P450, cytochrome b_5, NADPH reductase, and NADH reductase per mg microsomal protein does not change significantly with age. In addition, the electrophoretic profile of the microsomal peptides is unaltered with age (Birnbaum and Baird, 1978a).

Since the major protein components of the mixed function oxidase system remain constant with age, it has been proposed that the altered mixed function oxidase activity may be due to changes in the microsomal membrane with age (Birnbaum and Baird, 1978a). The purpose of the present study was to detect possible changes in microsomal membrane structure with age by measuring membrane fluidity using electron paramagnetic resonance (EPR) and spin labeling. It was found that there were significant changes in

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membrane fluidity with age. It is proposed that these fluidity changes may account for some of the previously reported changes in mixed function oxidase activities with age.

MATERIALS AND METHODS

Male CFN rats, a Wistar-derived inbred strain, were used in all experiments. They were bred and maintained as previously described (Baird *et al.*, 1975). Food was removed from rats 18 h prior to sacrifice, and the rats were sacrificed by decapitation and exsanguination. Hepatic microsomes were prepared by differential centrifugation from uninduced rats aged 3 months (young), 12 months (middle-aged), and 24–27 months (old) as described previously (Birnbaum *et al.*, 1976). About 100 mg of the final microsomal pellet was resuspended in 0.3 ml of 50 mM Tris-HCl (pH 7.5), 50% glycerol, and 0.01% BHT. The samples were gassed with nitrogen, quick frozen in a dry ice/propanol bath, and stored at −70°C until use. Samples were used within one month of preparation. Microsomal membranes prepared from each age group by this method have very similar levels of microsomal mixed function oxidase components (Birnbaum and Baird, 1978a). In addition, the electrophoretic profile of microsomal peptides is similar. The ratio of total lipid to microsomal protein is also constant with age in these membrane preparations (Birnbaum and Baird, 1978b).

Microsomal membranes were spin-labeled by equilibration with a solution containing a spin label/bovine serum albumin complex (Hubbell and McConnell, 1969). Prior to spin labeling, membrane samples were thawed and washed once with 100 mM potassium phosphate buffer (pH 7.8). The spin label/bovine serum albumin solution was prepared by drying down 2 mg of spin label dissolved in chloroform onto the bottom of a 25 ml Erlenmeyer Flask and adding 2 ml of 5% bovine serum albumin (essentially fatty acid free, crystallized and lyophilized, Sigma Chemical Company, St. Louis, MO). The solution was equilibrated overnight with shaking in a water bath. One ml of spin label/bovine serum albumin solution was mixed with 1 ml of microsomes (about 4 mg microsomal protein). After 1 h, the labeled microsomes were washed 3 times in the phosphate buffer to remove label that was not incorporated into the microsomes. EPR analysis of the final wash revealed no trace of the spin label/bovine serum albumin complex. After the final wash, the microsomes were resuspended in 0.150 ml of phosphate buffer by homogenization with a glass-Teflon ® homogenizing system. This spin-labeling procedure resulted in a spin label-to-membrane lipid ratio of less than 1:100. This ratio produces almost no alteration of red blood cell membranes (Butterfield *et al.*, 1976). The spin labels used in this study were the 5-nitroxide stearic acid (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl) and the 16-nitroxide stearic acid (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl) purchased from Syva Company (Palo Alto, CA).

The spin-labeled microsomal membranes were examined using a Varian E109E EPR spectrometer (Varian Associates, Palo Alto, CA). The membrane suspension was drawn up into a 0.05 ml capillary tube, the capillary tube was placed in an EPR quartz sample tube, and both tubes placed in the EPR spectrometer. Spectra were recorded at X-band frequency using 100 kHz modulation. The microwave power was 5 mW, modulation amplitude was 2 G, scan range was 100 G, and microwave frequency was 9100 MHz for all samples. The sample temperature was maintained using a Varian variable temperature accessory. Temperature was calibrated to within 0.2°C using a copper/constantan thermocouple encased in teflon and connected to a digital thermometer (Bailey Instrument Co., Saddle Brook, NJ). Samples were allowed to equilibrate at a given temperature for 5 min before their spectra were recorded.

The fluidity of the labeled membranes was quantitated using the order parameter S as described by Hubbel and McConnell (Hubbel and McConnell, 1971). For membranes labeled with the 5-nitroxide label, the T-tensor components $T_{\parallel}'$ and $T_{\perp}'$ were calculated from the formula $A = 2\,T_{\perp}' - 1.6$ and $B = 2\,T_{\parallel}'$, where A and B are the distances shown in Figure 1. The isotropic coupling constant a' was defined as $a' = (2\,T_{\perp}' + T_{\parallel}')/3$ and $S = 0.564\,(T_{\parallel}' - T_{\perp}')/a'$. For membranes labeled with the 12-nitroxide label, only $T_{\perp}'$ was calculated.

Date is expressed as mean ± standard error (SE) in experiments where the membrane fluidity of individual animals was determined (Table 2). Statistical significance was determined using Student's unpaired *t*-test (Dixon and Massey, 1969). The temperature-fluidity data (Figure 2) was fitted to two linear least squares lines, one from 18 to 24°C and the other from 24 to 31°C. To determine points of change in the slope of the temperature-fluidity curve, a least squares analysis was used. The slope at a given temperature was determined from a 5-point linear least squares line. The slope at each point was then compared with its neighbor for significant differences.

RESULTS

The fluidity of microsomal membranes isolated from rats aged 3, 12, and 26 months was compared at room temperature using the 5-nitroxide and 16-nitroxide stearic acid spin labels (Table 1). The 5-nitroxide spin label demonstrated a decrease in S with increasing age. This indicated an increase in the mobility of the spin label with age. The

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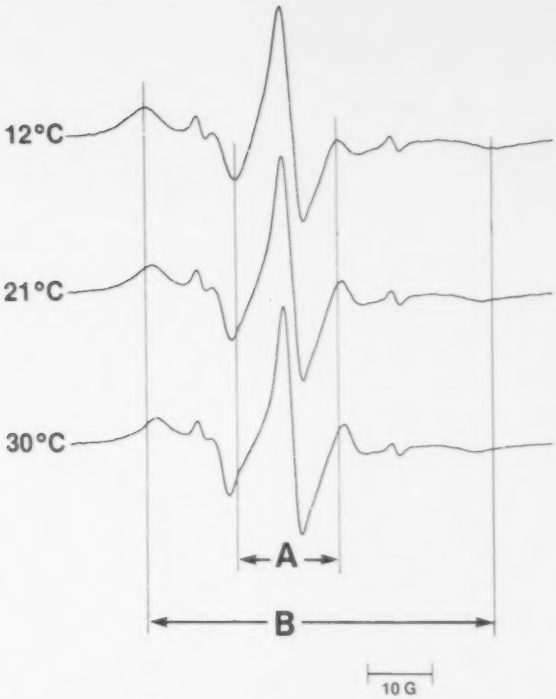

FIG. 1. Effect of temperature on EPR spectrum of spin-labeled hepatic microsomal membranes from old rats. Membranes were isolated from 26 mo old rats and labeled with 5-nitroxide spin label. Distances A and B were used to calculate the isotropic coupling constant and the order parameter (see Materials and Methods).

16-nitroxide spin label also showed an increase in mobility with age, since $T_{\perp}'$ increased with age. The isotropic coupling constant a′ decreased with age. This indicated an increase in the hydrocarbon environment of the spin label with age.

Membrane fluidity of membranes isolated from individual rats was studied as a function of age and temperature. As expected, the EPR spectrum of microsomal membranes labeled with the 5-nitroxide label demonstrated increased fluidity with increased temperature (Figure 1). With increasing age, there was a progressive increase in membrane fluidity at each temperature studied (Table 2). There was a significant difference between the order parameter S of young and old membranes at all temperatures studied. However, there was no significant difference between young and middle-aged membranes. At 12°C and 30°C, there was a significant difference between the order parameter of middle and old membranes. These results demonstrated an increase in membrane fluidity during aging (12 to 27 months) that was as great or greater than that during maturation (3 to 12 months). The isotropic coupling constant a′ also progressively decreased with age, but the changes were only significant at 12°C (Table 2). This indicated an increase in the hydrocarbon nature of the environment around the spin label with age.

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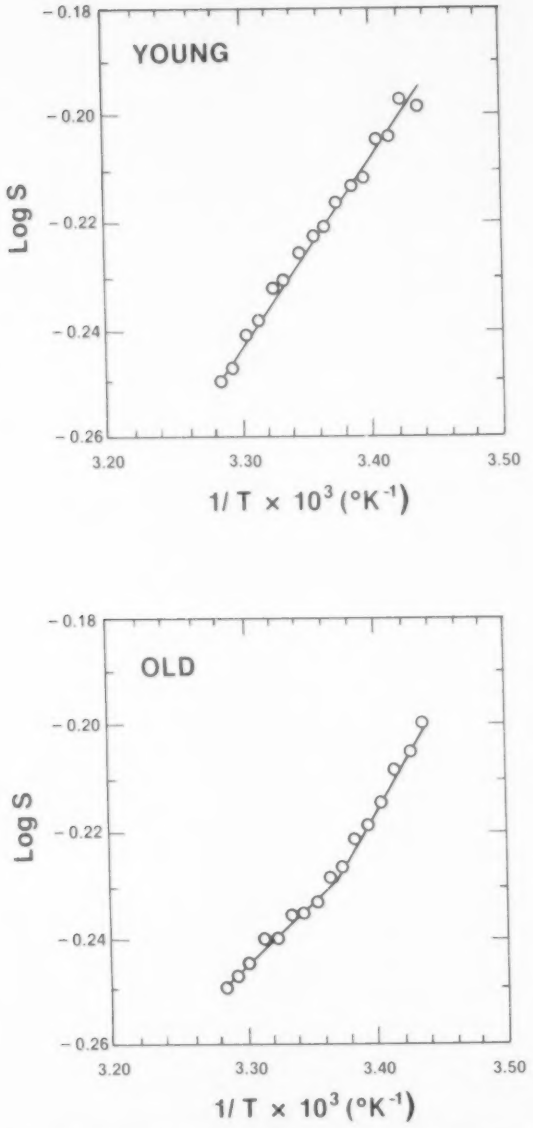

FIG. 2. Change in microsomal membrane fluidity with temperature. Membrane fluidity, as measured by the order parameter S, was determined from 18–31°C in steps of 0.9°C. Microsomal membranes from 4 young (3 mo) and 4 old (26 mo) rats were pooled and labeled with 5-nitroxide spin label. Each point is the average of 3 determinations. The regression lines for the young was $y = 0.357 \times -1.42$ (18°–31°C). The regression lines for the old were $y = 0.419 \times -1.64$ (18°–24°C) and $y = 0.242 \times -1.04$ (24°–31°C).

TABLE 1. EFFECT OF AGE ON MEMBRANE FLUIDITY

Age (mo)	a'* (gauss)	S*	$T_{\perp}'$** (gauss)
3	15.20	0.629	12.4
12	15.16	0.612	12.5
26	15.07	0.606	12.6

Microsomal membranes from 4 rats of each age group were pooled and labeled with either 5-nitroxide or 16-nitroxide spin label. Fluidity was measured at room temperature.

*Measured using 5-nitroxide spin label.

**Measured using 16-nitroxide spin label.

A detailed study was made of membrane fluidity in the temperature range of 18 to 31°C using pooled membrane samples (Figure 2). In the old membranes, a significant change in slope was detected at 24°C when the log of the order parameter was plotted against reciprocal temperature. The slope of the line above 24°C was significantly different ($p < 0.001$) than the slope of the line below 24°C (0.242 ± 0.021 vs. 0.419 ± 0.011, mean ± S.D.). On the other hand, the relationship between fluidity and temperature in the young membranes was most simply described by a single straight line from 18°-31°C. There was no evidence of a change in slope at 24°C in the young membranes.

DISCUSSION

These studies demonstrate organizational change in the lipid component of the microsomal membrane between 3 and 27 months. This is evidenced by the general increase in membrane fluidity with age (Table 2) and by differences in the temperature-fluidity relationship of young and old membranes (Figure 2). The increase in membrane fluidity with age was seen with both the 5-nitroxide and 16-nitroxide spin labels (Table 1). This indicated that the increased fluidity extended from the surface of the membrane to the in-

TABLE 2. EFFECT OF AGE AND TEMPERATURE ON FLUIDITY OF MEMBRANES FROM INDIVIDUAL RATS

Parameter measured	Temperature	Age		
		3 mo	12 mo	26 mo
S	12°C	0.689 ± 0.003	0.671 ± 0.007	0.652 ± 0.003[a,b]
S	21°C	0.627 ± 0.005	0.609 ± 0.006	0.605 ± 0.001[a]
S	30°C	0.586 ± 0.003	0.581 ± 0.002	0.569 ± 0.003[a,b]
a'	12°C	15.34 ± 0.05	15.22 ± 0.02	15.17 ± 0.01[a]
a'	21°C	15.18 ± 0.04	15.12 ± 0.15	15.11 ± 0.01
a'	30°C	15.14 ± 0.04	15.08 ± 0.03	15.07 ± 0.03

Table entries are the mean ± SEM of 4 rats. Microsomes from each rat were individually labeled with the 5-nitroxide spin label, and the parameters were determined at the indicated temperatures. S is a dimensionless parameter and the units of a' are gauss.

[a]Significantly different than 3 mo ($p < 0.05$).

[b]Significantly different than 12 mo ($p < 0.05$).

terior. Previous studies have reported a sharp increase in microsomal membrane fluidity at birth and a continued increase from birth to 3 months (Kapitulnik *et al.*, 1979). The experiments reported here extend these studies and show a further increase in fluidity from 3 to 25–27 months.

Membrane fluidity is influenced by many factors including the phospholipid to cholesterol ratio and the degree of saturation of the phospholipid acyl chains (Thompson and Huang, 1978). Previous compositional studies of the microsomal membranes from rats identical to those used in these experiments showed a decrease in the ratio of phospholipid to lipid with age (Birnbaum and Baird, 1978b). In model systems, a decrease in the phospholipid to lipid ratio results in a decrease in fluidity (Cullis, 1976). The fact that microsomal membranes show an increase in fluidity with age despite a decrease in the phospholipid to lipid ratio indicates that other compositional changes may occur with age. One possibility is that the degree of unsaturation of the phospholipid acyl chains increases with age. In model systems, membrane fluidity increases as the degree of unsaturation increases (Lentz *et al.*, 1976). In support of this hypothesis, analysis of total lipid extracts from liver microsomes from male Sprague Dawley rats showed that there is an overall increase in the degree of unsaturation of the major fatty acids with age (Grinna, 1976). This is due primarily to a large increase in the percentage of 22:6 found in the membranes of the adult rats.

The second line of evidence for a structural change in the microsomal membrane with age is the differences in the temperature-fluidity relationship between young and old (Figure 2). No break in the temperature-fluidity was observed with young membranes over the temperature range studied (Figure 2). This is consistent with a calorimetric study of microsomal membranes from young rats (Mabrey *et al.*, 1977). That study found no evidence for a reversible lipid phase transition above 0°C, indicating that the microsomal membrane is in a fluid state. In the present study, a significant change was seen in the slope of the temperature-fluidity plot of the old membranes at 24°C (Figure 2). Such changes of slope are usually interpreted as evidence of lipid phase transitions in certain regions of the membrane (Wisnieski *et al.*, 1974). Above 24°C, the old membranes were more resistant to the fluidizing effect of temperature than were the young membranes. This may reflect the decrease in the phospholipid to lipid ratio of the microsomal membranes with age (Birnbaum and Baird, 1978b). In lean mice, the microsomal membrane exhibits a phase transition at 22.5°C (Rouer *et al.*, 1980). This phase transition is not detectable in membranes from obese and diabetic mice. Microsomal membranes from lean mice were shown to contain a greater percentage of unsaturated fatty acids compared to membranes from obese and diabetic mice.

It has been shown that the activity of many membrane-bound enzymes may be modulated by the fluidity of the membrane as determined by its composition (Sandermann, 1978). The cytochrome P450 mixed function oxidase system may be especially sensitive to membrane fluidity since it requires the interaction of several separate membrane-bound proteins to function. Several studies have reported correspondences between cytochrome P450 function and membrane phase transitions (Rouer *et al.*, 1980; Eletr *et al.*, 1973; Narasimhulu, 1978). In addition, P450 functions may be altered by feeding diets which change the microsomal membrane composition and therefore its fluidity (Mabrey *et al.*, 1977; Becker *et al.*, 1978). Therefore, the changes in microsomal membrane fluidity with age reported in this study may contribute to the observed decrease in certain mixed

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function oxidase reactions in the rat with age. Other factors may also be important in determining changes in P450 functions with age, however. This is demonstrated by the fact that some P450 enzyme activities in the CFN rat increase with age (Birnbaum, 1979) and some remain unchanged (Baird *et al.*, 1975).

Changes in membrane fluidity with age may contribute to the overall decline in organ function seen in aging animals. Changes in the viscosity of the lymphocyte plasma membrane with age has been implicated in the age-related decrease in immune function (Rivnay *et al.*, 1979). In mitochondria from the heart, there are correlations between the decrease in the specific activity of certain enzymes and changes in the mitochondrial membrane with age (Nohl, 1979). The data reported here suggest that changes in microsomal membrane fluidity may account for some of the age-related changes in mixed function oxidase activity of the liver.

SUMMARY

There are changes in the mixed function oxidase enzymatic activities of rat hepatic microsomal membranes with age. However, the protein components of the mixed function oxidase system do not appear to change with age. The purpose of this study was to detect possible changes in the fluidity of the lipid component of the microsomal membrane with age. Hepatic microsomes were isolated by differential centrifugation from uninduced, male CFN rats aged 3, 12 and 26 mo. The microsomal membrane fluidity was measured using electron paramagnetic resonance after incorporation of a 5-nitroxide stearic acid spin label into the membrane. The order parameter S decreased with age from 0.586 ± 0.003 (3 mo) to 0.581 ± 0.002 (12 mo) to 0.569 ± 0.003 (26 mo) at 30°C. This indicated an increase in membrane fluidity with age. In membranes labeled with the 16-nitroxide stearic acid, a similar increase in membrane fluidity with age was observed. The order parameter of microsomal membranes from 3 and 26 mo rats was measured over the temperature range 18° to 31°C in steps of 0.9°C. A plot of the log of S versus the reciprocal temperature revealed a phase transition at 24°C in membranes from 26 mo rats, but no phase transition was observed in 3 mo old rats in this temperature range. The change in fluidity of the hepatic microsomal membrane with age may account for some of the observed changes in membrane-bound mixed function oxidase activities with age.

Acknowledgement—The authors gratefully acknowledge the excellent technical assistance of Mr. Britt Thomas and the secretarial assistance of Mrs. Sandy Melliere. This research was supported in part by the Veterans Administration.

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Experimental Gerontology, Vol. 17, pp. 49–64, 1982
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0531-5565/82/010049-16$03.00/0
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INFLUENCE OF WEANING-INITIATED DIETARY RESTRICTION ON RESPONSES TO T CELL MITOGENS AND ON SPLENIC T CELL LEVELS IN A LONG-LIVED F_1-HYBRID MOUSE STRAIN

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(*Received* 22 *March* 1981)

INTRODUCTION

FOOD RESTRICTION (but without malnutrition) initiated early in life and continued until death increases mean and maximal lifespans in rats (McCay *et al.*, 1935; Ross, 1961), mice (Tucker, 1979; Cheney *et al.*, 1980), fish (Comfort, 1963), rotifers (Fanestil and Barrows, 1965), and the protozoan *Tokophyra* (Rudzinska, 1962). As discussed elsewhere (Walford, 1969), an extension of maximal survivorship of already genetically long-lived animal strains involves an interaction with mechanisms basic to the aging process, as opposed merely to optimization of the environment. Food restriction increases maximal lifespan more potently and consistently than any other (usually pharmacologic) procedure tested in rodents (Walford, 1969; Ross, 1978; Cutler, 1981). Likewise, dietary restriction appears to be the sole strategy yet tested in homeotherms which slows age-related increases in mortality rates, i.e., which changes the slope of the mortality curve (Sacher, 1977). The rodent work has typically involved food restriction beginning at weaning (3–4 weeks of age), and appears to depend on restricting calories while providing adequate intakes of other dietary essentials. Associated with the increased survivorship are a reduced incidence and delayed onset of late-life spontaneous cancer and other diseases in underfed rodents (Tannenbaum, 1942; Berg and Simms, 1960; Ross and Bras, 1965). Thus appropriately engineered dietary restriction represents an important model-tool to study decelerated mammalian aging.

It has been our view that immunologic consequences of food restriction might contribute to the effects on survivorship and disease. The immune systems of mammals show senescent changes which might be involved in either the etiology or pathogenesis of aging (Walford, 1969, 1974). These alterations (reviewed by Weindruch and Walford, 1981) have been documented in both mice and humans with findings suggesting two types of change. A *decreased* capacity to respond to exogenous stimuli (*e.g.*, T cell mitogens such as phytohemagglutinin (PHA) and concanavalin A (Con A); sheep red blood cells; al-

loantigens) occurs after puberty, along with an *increase* in autoimmunity. Thymus dependent exogenous responses appear more altered than thymus-independent ones, which probably reflects age-dependent changes in thymic structure (Hirokawa, 1978) and differentiative capacities (Hirokawa and Makinodan, 1975; Hirokawa and Sado, 1978). A particular T cell emphasis also fits with our proposal that the major histocompatibility complex, and genes linked thereto, such as the gene for mitochondrial superoxide dismutase, comprise an important life-support homeostatic system (Walford *et al.*, 1981).

Life prolonging, weaning-initiated underfeeding of mice profoundly influences age-sensitive immunologic indices. In previous studies with genetically long-lived mouse strains such as C57BL/6J and C57BL/10Sn × C3H/HeDiSnF_1 (abbreviated as B10C3F_1), old mice restricted since weaning showed more vigorous spleen cell responses than age-matched controls to injected sheep red blood cells and to stimulation by T cell mitogens (Gerbase-DeLima *et al.*, 1975; Mann, 1978; Weindruch *et al.*, 1979). However, existing data are conflicting with respect to nutritional effects on splenic T cell mitogen responses in early life, since reduced responses were reported for C57BL/6J mice either 2 months of age (Mann, 1978) or 6–8 months of age (Gerbase-DeLima *et al.*, 1975), versus elevated responses for diet restricted 3 month old C3H mice (Fernandes *et al.*, 1976), 7 month old C57BL/6J mice (Mann, 1978), and 9 month old B10C3F_1 mice (Weindruch *et al.*, 1979). C57BL/6J mice restricted by intermittent feeding showed elevated splenic PHA and Con A reactivity when assayed the day after being fed, compared to 2–3 days post-feeding (Mann, 1978). Thymuses from diet restricted B10C3F_1 showed stunted growth in weight (Weindruch *et al.*, 1979), and thymuses from 6 month old underfed mice appeared histologically less mature than those from age-matched controls (Weindruch and Suffin, 1980). Late-life increases in autoimmunity were inhibited by weaning-initiated food restriction (Friend *et al.*, 1978; Barnett *et al.*, 1981). These various "rejuvenating" immunologic effects contrast sharply to the negative impacts of diverse malnutritional states.

The present studies were undertaken to clarify certain aspects of weaning-initiated underfeeding on age-sensitive immune responses in a long-lived mouse hybrid. We have focused on proliferative responses to T cell mitogens. We observed that "undernutrition without malnutrition" decreased the number of nucleated cells recovered per spleen but increased splenic PHA reactivity, while inconsistently increasing responses to Con A. In view of both the strong sensitivity of the PHA response to be reduced by aging and increased by underfeeding, further analysis was carried out. An increase was observed for underfed mice in the percentage of splenic T cells and of PHA-induced splenic blast cells. PHA responses of underfed mice were greater even as limiting cell dilutions were approached. Neither suppression nor synergy were detectable in PHA-stimulated mixed cultures of spleen cells from control and restricted mice. Lymph node cell responses to PHA were not influenced by underfeeding. These observations provide evidence that food restriction raises splenic PHA reactivity at least in part by increasing the proportion of PHA responsive T cells.

METHODS AND PROCEDURE

Mice

(C3H.SW/Sn × C57BL10.RIII/Sn)F_1 mice (abbreviated C3B10RF_1), bred in our animal facilities, were used. This parental combination was selected on the basis of previous studies suggesting its potential for gen-

erating a long-lived mouse hybrid susceptible to a variety of spontaneous neoplasms (Smith and Walford, 1979), while showing typical age changes in maturation and senescence of immune capacities (Meredith and Walford, 1977). Studies currently in progress indicate that females fed Purina Lab Chow® *ad libitum* show a mean lifespan of 27–29 months. Females were used in all experiments and males in only one experiment. All mice consuming semi-synthetic diets were housed individually in plastic cages, whereas males fed Purina Lab Chow® *ad libitum* were caged in groups of 4–6. A group of females fed Purina Lab Chow® *ad libitum* was housed individually and studied only for growth rates, not for immune responses. They differed from other groups in that they were suckled in litters of 1–4 mice, whereas other cohorts were not selected on the basis of litter suckling size. All experiments involved mice 15 months of age or younger, the 15 months corresponding to mid-adulthood.

Composition of diets and feeding strategies

Dietary restriction was instituted at weaning (21–24 days of age). Table 1 shows the composition of the diets used. These nearly isocaloric formulations were prepared by us once monthly and stored at 4°C. Diet 1 was fed to control females (designated N for "normally" fed) in specified amounts producing a growth response greater than restricted mice but less than that of mice freely fed Purina Lab Chow® (designated LC). Group N mice received 85 Cals. per week. Diet 2 was given in restricted quantities via intermittent feeding. Restricted mice are designated R_{40} or R_{50} with the subscript indicating Cals. fed per week. Diet 2 was enriched relative to Diet 1 in casein, salts, vitamin mixture and brewer's yeast such that the per week intake of these nutrients for restricted mice approximated that of mice consuming Diet 1. In contrast, weekly intakes of carbohydrate and fat (corn oil) were less for underfed mice than for control animals consuming Diet 1. Males fed LC *ad libitum* were used only in the initial immunologic experiment and all subsequent comparisons involved female mice consuming known amounts of semipurified diets.

Cell preparation and culture

Mice were killed by cervical dislocation. Unless otherwise noted, all cultures were initiated on the day after feeding of restricted mice (*i.e.* Tuesday or Thursday) and always involved study of individual rather than pooled spleens. The spleens were asceptically removed, placed in cold media (RPMI-1640 [Microbiological Associates, Los Angeles, CA]) supplemented with penicillin, streptomycin, fungizone and 25mM HEPES buffer and gently pressed through a sterile aluminum mesh screen with a tuberculin syringe plunger. The cell suspensions were washed once and erythrocytes lysed with 7% ammonium chloride. The suspensions were washed three times in media and were adjusted to a concentration of 6×10^6 viable cells per ml in 95% RPMI + 5% heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Santa Clara, CA). Cell viability ranged from 80–95% as judged by trypan blue exclusion. When lymph node lymphocytes were tested, pooled brachial, inguinal and axillary lymph nodes were processed as above but without the ammonium chloride treatment and subsequent washes.

TABLE 1. COMPOSITION OF DIETS (GRAM/KG DIET)

Constituents	Diet 1[a]	Diet 2[b]
Casein, vitamin free test[c]	200.0	350.0
Cornstarch	260.8	157.6
Sucrose	260.8	157.6
Corn oil (Mazola®)	135.0	135.0
Nonnutritive fiber	56.4	40.0
Salt mixture, USP XIV	60.0	110.0
Vitamin mixture	23.0	42.2
Brewer's yeast	4.0	7.4
Zinc oxide	0.05	0.1

[a]Diet 1: Diet fed to control mice (Group N) as seven 3.0–3.2 gm feedings per week (one daily feeding on Monday through Thursday, 3 feedings on Friday) providing about 85 Cal./wk. All food was regularly consumed.

[b]Diet 2: Diet fed to restricted mice. 35% casein diet, enriched also in salt and vitamin mixtures, Brewer's yeast and zinc oxide. Fed as four 2.5–2.7 gm or 3.0–3.2 gm feedings per week (one daily feeding on Monday and Wednesday, 2 feedings on Friday), which respectively provided about 40 Cal./wk or 50 Cal./wk.

[c]These ingredients were purchased from ICN Pharmaceuticals (Cleveland, OH): casein, fiber, salt mixture, vitamin mixture and brewer's yeast. Cornstarch, sucrose and Mazola® were purchased locally.

A microculture method described by Meredith and Walford (1977) was used to assay mitogen-induced DNA synthesis. Cell suspensions (6×10^5 in 0.1 ml) were added to microtiter plates (Scientific Products, Santa Ana, CA) along with either 0.1 ml of RPMI + 5% FCS alone or with the mitogen. Mitogen doses used, 2.5 μl/ml for PHA (PHA-P from Difco Labs, Detroit, MI), 2.0 μg/ml for Con A (Miles-Yeda, Ltd., Kankakee, IL), 30 μl/ml for pokeweed mitogen (PWM, Grand Island Biological Co.) and 350 μg/ml for lipopolysaccharide (LPS) (WO55.B5 from Difco Labs., Detroit, MI), were those found to optimally stimulate spleen cells from 3–4 month old LC males. Rigorous mitogen dose-response studies were not performed with cells from restricted mice as optimal concentrations have been reported not to differ between normal control and restricted mice (Mann, 1978). Additionally, using mitogen doses optimal for normals, food restricted mice showed higher responses than the normals. Triplicate cultures were carried out for each mitogen-stimulated and control test. Results are reported as (cpm in stimulated cultures) − (cpm in control cultures). Optimal incubation periods for spleen cells from normally-fed mice were 48 hours except for LPS which required 72 hours. Cultures were labeled for the last 24 hours with 2 μCi of tritiated thymidine (^{3}H-TdR, 1.9 C/M Mole activity, Schwarz-Mann, Orangeburg, NY) in 0.05 ml media/well. In some experiments cell doses as well as culturing and labeling times were varied.

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Cell dosage and mixing experiment

Normal and R_{50} mice of 13–14.5 months of age were compared regarding cell dose-response to PHA by varying the number of responder cells plated ($1.2–6 \times 10^5$/well). A total of four mice of each group were tested in two experiments. Mixing experiments were performed with the cells from the same animals. Splenocytes from N and R were combined in varying proportions (80%N:20%R, 50N:50%, 20N:80R) to equal a total of 6×10^5 cells/well. Observed values were compared to expected. Expected values were calculated on the basis of the observed values for 6×10^5 cells of each population alone. For example, if 6×10^5 cells from one N mouse gave a response of 20,000 cpm, cells from this spleen when forming 80% of a mixed culture (4.8×10^5 cells) would be expected to provide (.8) (20,000 cpm) = 16,000 cpm.

PHA-induced blast cell quantitation

Spleen cell suspensions were incubated in microtiter plates with PHA or 95% RPMI − 5% FCS as described above. After 48 hours the contents of the wells were pipetted into tubes, cytocentrifuged, and stained with Wright's stain. One hundred cells were classified as blasts or non-blasts. Blast cells were defined as large cells with various amounts of dark blue cytoplasm, large nuclei with fine chromatin pattern and prominent nucleolus. Non-blast cells were characterized as lymphoid cells with scanty blue cytoplasm, small nucleus and coarse chromatin. Results are reported as (% blast cells in PHA-stimulated cultures) − (% blast cells in unstimulated cultures).

T cell quantitation

Two methods were used to quantitate the proportions of T cells in the lymphoid cell suspensions. One measured cytotoxicity using the protocol and reagents (anti-mouse T cell serum and rabbit complement [C']) from Accurate Chemical and Scientific Co. (Hicksville, NY). The percent specific cytotoxicity was calculated as (% cells killed by anti-T cell serum + C') minus (% cells killed by C' alone). T cells were also measured by indirect immunofluorescence using monoclonal anti-mouse Thy 1.2 (a mouse IgM molecule from New England Nuclear, Boston, MA) and fluorescein conjugated $F(ab')_2$ fragment goat anti-mouse IgM (abbreviated as F*) (Cappel Laboratories, Cochranville, PA). The cells (0.2 ml at 10^7 cells/ml) were incubated on ice for 25 minutes with 0.05 ml anti-Thy 1.2 such that the final antibody dilution was 1:1000. This dilution was selected on the basis of product specifications showing maximal cytolysis of T cells through about a 1:10,000 dilution. Control cultures were incubated with 0.05 ml PBS. The cells were then washed twice in media, F* (0.05 ml diluted 1:10) was added and the combination was incubated on ice for 45 minutes. The cells were next resuspended in 1.0 ml RPMI, layered on top of 3.0 ml of 5% bovine serum albumin (Miles, Elkhart, IN) −95% RPMI and washed to remove debris. Layering was repeated, slides were made, coded, and scored by two observers using a Leitz Ortholux microscope.

Statistics

Values represent determinations on individual animals (*i.e.* no pooling) unless otherwise noted. Intergroup differences were analyzed by the two tailed t statistic for two means. The cell mixing experiment was analyzed via a one sample t test comparing the observed:expected ratios to unity.

RESULTS

Body weight gain

Influences of food restriction on post-weaning body weight gain are shown in Figure 1. The values were graphed as $\overline{X} \pm SD$ (instead of $\overline{X} \pm SE$, which was used for all other data presented) so as to provide a clearer view of variance in these large (n = 45–72) cohorts. Food restriction slowed growth and reduced peak body weight. Most $C3B10RF_1$ mice given free access to Purina Lab Chow® become moderately obese, an observation in line with LC ♀ adult body weights of about 45 g (Fig. 1) and LC ♂ of about 50 g (data not shown). LC ♀ and LC ♂ became heavier than cohort N, signifying that the control mice (cohort N) used for almost all immunologic testing were restricted dietarily relative to *ad libitum* fed LC ♀ and LC ♂. At the mid-adulthood age of 14–18 months, the weights of N, R_{50} and R_{40} mice were respectively 80%, 46% and 38% of the mean body weight for LC ♀.

Initial testing of spleen cell mitogen responses

As noted, some confusion exists regarding the influence of weaning-initiated underfeeding on *in vitro* mitogen-induced spleen cell proliferation in young mice. Table 2 shows

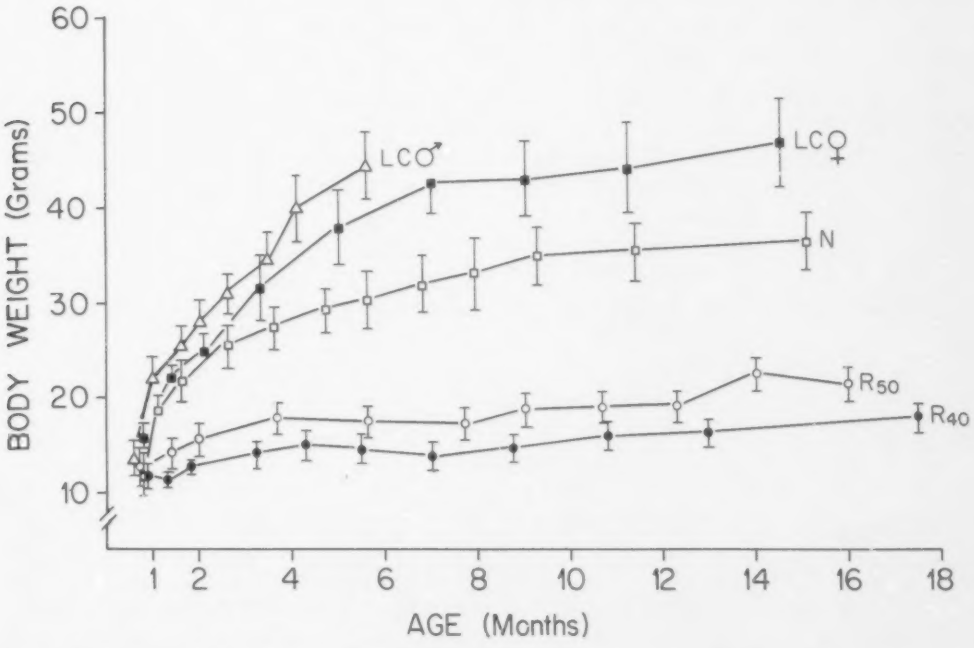

FIG. 1. Body weight gain in dietarily restricted and control mice. Values represent $\overline{X} \pm SD$ for $n = 48$ (LC ♂), $n = 45$ (LC ♀), $n = 57$ (N), $n = 72$ (R_{50}) and $n = 55$ (R_{40}). Body weights of LC ♀ vs. N vs. R_{50} vs. R_{40} were highly significantly different at all but very early ages. For example, 14.5 month old LC ♀ mice were heavier than 15.1 month old N mice ($P < 0.001$) and 16.0 month old R_{50} mice outweighed 17.5 month old R_{40} mice ($P < 0.001$).

TABLE 2. SPLEEN CELL MITOGEN-INDUCED PROLIFERATIVE RESPONSES IN 3½–4 MONTH-OLD DIETARILY RESTRICTED AND CONTROL MICE[a]

Group (n)[b]	PHA	Con A	PWM	LPS
LC ♂ (6)	64,993 ± 4470	140,961 ± 3969	12,980 ± 2282	44,666 ± 1972
R_{50} (4)	87,425 ± 9522[c]	211,961 ± 24,202[d]	22,375 ± 9359	40,960 ± 6494

[a]Values represent $\bar{X}$ ± *SE* of responses of individual mice. Cultures were done in triplicate and are expressed as cpm ^{3}H-TdR corrected for unstimulated uptake (which ranged from between 1000–6000 cpm and was not influenced by underfeeding).

[b]Number tested. The LC ♂ mice were the only males used in any experiment.

[c]Significantly different from LC ♂ with $P < 0.05$.

[d]Significantly different from LC ♂ with $P < 0.01$.

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spleen cell proliferative responses to PHA, Con A, PWM and LPS for 3½–4 month old LC ♂ (n = 6) and R_{50} (n = 4) mice. When compared to LC ♂, R_{50} mice displayed reduced body weights (18.1 ± 0.7 g *vs.* 40.6 ± 2.4 g (SE)), spleen weights (46 ± 4 mg *vs.* 86 ± 5 mg) and spleen cell yields (38 ± 7 × 10^6 vs. 76 ± 7 × 10^6). R_{50} mice showed significantly greater responses to PHA ($P < 0.05$) and Con A ($P < 0.01$) than LC ♂ mice, while PWM and LPS responses did not differ between groups. Unstimulated ^{3}H-TdR uptake for 48 or 72 hours of culture was not influenced by diet.

Effects of the fasting interval on spleen cell PHA and Con A reactivity

To confirm and extend Mann's observations (1978) on the impact of the fasting interval on mitogen induced lymphocyte proliferative responses, the following experiments were carried out. Three month old N and R_{40} mice were killed on either Wednesday morning (the "fasting cycle" for R_{40}, which were last fed on Monday morning) or on Thursday morning ("feeding cycle" for R_{40}, last fed on Wednesday morning). Mice so treated are noted as R_{40}-Fasted and R_{40}-Fed. Cellular ^{3}H-TdR uptakes subsequent to PHA or Con A stimulation appear in Table 3. PHA responses of splenocytes from R_{40}-Fed mice exceeded responses of both N ($P < 0.001$) and of R_{40}-Fasted mice ($P < 0.02$). PHA responses of

TABLE 3. EFFECT OF FASTING INTERVAL ON SPLEEN CELL PHA AND CON A RESPONSES IN 3 MONTH-OLD DIETARILY RESTRICTED AND CONTROL MICE[a]

Group (n)[b]	PHA	Con A	Unstimulated
N (8)	59,000 ± 5238[c]	177,270 ± 8314	3048 ± 264
R_{40}-Fed (6)	112,950 ± 5555	201,090 ± 12,069	2476 ± 367
R_{40}-Fasted (7)	85,626 ± 7647	135,706 ± 18,670	970 ± 258

[a]PHA and Con A values represent $\bar{X}$ ± *SE* for individual mice expressed as cpm ^{3}H-TdR corrected for unstimulated uptake.

[b]R_{40}-Fed represents R_{40} mice sacrificed on a Thursday morning (24 hours after being fed). R_{40}-Fasted represents R_{40} mice sacrificed on a Wednesday morning (48 hours after being fed). Cultures were labelled at 24 hrs and harvested at 48 hrs. Determinations were for individual spleens except for 2 in each of the R_{40} subgroups which, because of too few cells, necessitated pooling 2 spleens.

[c]Statistically significant comparisons were: PHA – R_{40}-Fed > N ($P < 0.001$); R_{40}-Fed > R_{40}-Fasted ($P < 0.02$); R_{40}-Fasted > N ($P < 0.02$). Con A – R_{40}-Fed > R_{40}-Fasted ($P < 0.02$). Unstimulated-R_{40}-Fasted < N ($P < 0.001$); R_{40}-Fasted < R_{40}-Fed ($P < 0.01$).

R_{40}-Fasted exceeded those of N mice ($P < 0.02$). Fasted mice showed a reduction in Con A reactivity relative to R_{40}-Fed mice ($P < 0.02$) and a nearly significant decrease in Con A reactivity as compared to N mice ($0.10 > P > 0.05$). Unstimulated ^{3}H-TdR uptake was reduced in R_{40}-Fasted mice as compared to R_{40}-Fed ($P < 0.01$) or N ($P < 0.001$) animals.

Kinetic study

Does diet restriction alter PHA and Con A response kinetics? A two part kinetic study was carried out using first N *vs.* R_{50} mice and then N *vs.* R_{40} mice. Animals were 5–6 months old. PHA and Con A responses of spleen cells were determined with pooled spleens (2 spleens per pool for N and R_{50}; 4 spleens per pool for R_{40}). Labeling periods for the kinetic analysis were: 0 to 24 hours of culture (0→24h), 24→32h, 32→40h, 40→48h and 48→56h. Results appear in Figure 2. Clearly the N mice in each of the two experimental series responded in a consistent manner, allowing for combining of these data. Increasing the severity of food restriction tended to increase splenic responses to PHA

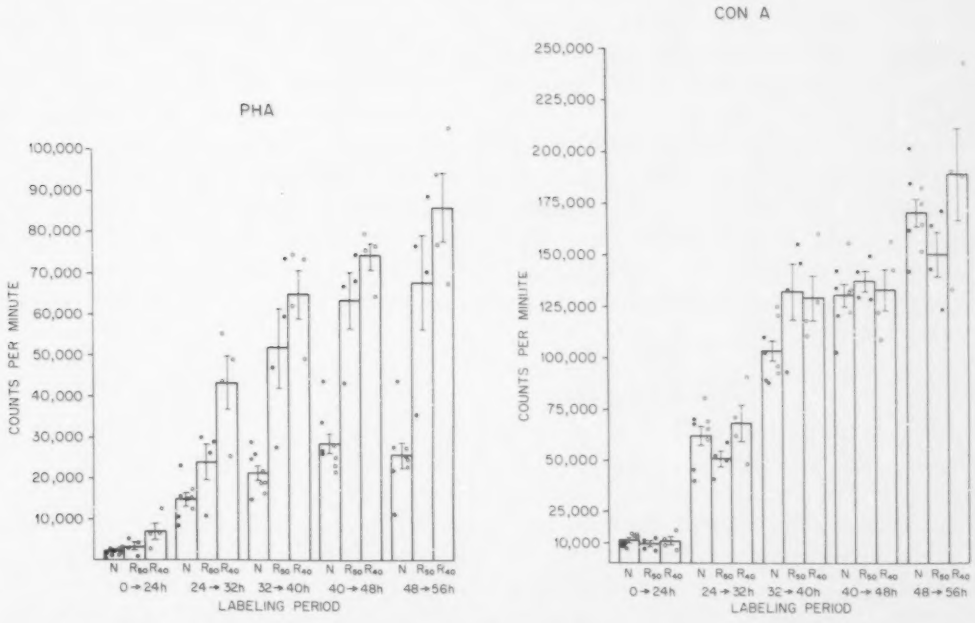

FIG. 2. Kinetic analysis of PHA (Fig. 2a) and Con A (Fig. 2b) reactivity of spleen cells from 5–6 month-old N, R_{50} and R_{40} mice. Values represent $\overline{X} \pm SE$. Each point represents ^{3}H-TdR uptake of cells from one pool of two spleens except for R_{40} which required four spleens/pool. Background cpm ranged from 500–4000, were not influenced by diet, and have been subtracted to give these values. Two series of experiments were carried out comparing first N *vs.* R_{50} and next N *vs* R_{40}. Individual mice studied in the N *vs.* R_{50} series are designated by •, whereas animals in the N vs. R_{40} series by o. Since responses of N mice in each series were consistent, these data were grouped for statistical analysis. Note that Y axes differ in scale by a factor of two between PHA and Con A. Statistically significant comparisons: PHA-R_{50} > N at 24→32h ($P < 0.05$), 32→40h ($P < 0.01$) and at both 40→48h and 48→56h ($P < 0.001$); R_{40} > N at 0→24h and 24→32h ($P < 0.01$) and for all subsequent periods ($P < 0.001$); $R_{40} > R_{50}$ at 24→32h ($P < 0.05$). Con A→R_{40} and R_{50} > N at 32→40h ($P < 0.05$).

(Fig. 2a) while generally not influencing Con A reactivity (Fig. 2b). PHA responses of R_{50} mice significantly exceeded those of N mice at 24→32h ($P < 0.05$), 32→40h ($P < 0.01$) and at both 40→48h and 48→56h ($P < 0.001$). PHA responses of R_{40} mice exceeded those of N mice at all labeling pulses with $P < 0.01$ at both 0→24h and 24→32h, and $P < 0.001$ for all subsequent periods. Average PHA responses of R_{40} mice consistently exceeded those of R_{50} mice but this difference was statistically significant only at 24→32h ($P < 0.05$). The kinetics of PHA reactivity for each group appeared similar with responses of R_{50} and R_{40} mice not increasing significantly after 32→40h (despite a tendency for an increase at 48→56h in R_{40}). The PHA response of N mice at 40→48h exceeded that of N mice at 32→40h ($P < 0.05$) but was not different from the response at 48→56h. Con A reactivity seemed generally unaltered by food restriction as the only statistically significant differences observed were at 32→40h, with responses of R_{50} and R_{40} exceeding those of N ($P < 0.05$). The highest average Con A responses were at 48→56h for each group, but the difference compared to the previous labeling period was significant only for N ($P < 0.001$).

One may note that the PHA responses of the 5–6 month old N mice in this kinetic study and in all subsequent experiments (all using mice 5 to 14.5 months old) averaged less than half that observed for 3–4 month old control mice in the two preceeding series. These differences might be related either to age-related decreases in PHA reactivity or to variations in the assay system occurring over the 18 months during which these studies were carried out. The strong PHA and Con A responses of R_{40} mice in this kinetic study argue against the latter possibility as affecting these particular data.

The increase in PHA reactivity of restricted mice was again associated with a sharp decrease in the number of nucleated cells recoverable from their spleens. Cell yield was determined for each spleen and $88 \pm 4 \times 10^6$ cells were recovered from group N spleens vs. $19 \pm 2 \times 10^6$ R_{50} cells recovered. Cell counts were not made on individual R_{40} spleens, but these contained even fewer cells as evidenced by the need to pool four spleens for each experiment.

Cell dosage and mixing experiments

Spleen cells from 13–14.5 month old N and R_{50} mice were stimulated with PHA in these experiments. Cell dose was reduced so that limiting dilutions would be approached and some insight gained about possible overt differences in PHA-responsive units. Mixing was done in an attempt to detect possible suppression by N cells or synergy by R_{50} cells. Spleen cell recovery was again much less in R_{50} ($26 \pm 4 \times 10^6$) than in N ($104 \pm 7 \times 10^6$) mice. Results are given in Figure 3, and show the effects of reducing the number of spleen cells per well responding to PHA. Average responses of R_{50} mice were greater at all doses tested and significantly so ($P < 0.05$) at the three highest doses (3×10^5, 4.8×10^5, 6×10^5 cells/well). Figure 4 gives results of coculturing N and R_{50} splenocytes. No statistically significant interactions were observed in this case.

PHA reactivity of lymph node lymphocytes

Does the elevation of PHA reactivity of spleen cell suspensions from underfed mice occur in other sites? To investigate this point we processed spleens and pooled peripheral lymph nodes from 12–13 month old N and R_{50} mice and tested for PHA reactivity. Long-term food restriction reduced cell yield in spleens ($32 \pm 4 \times 10^6$ cells per spleen for R_{50}

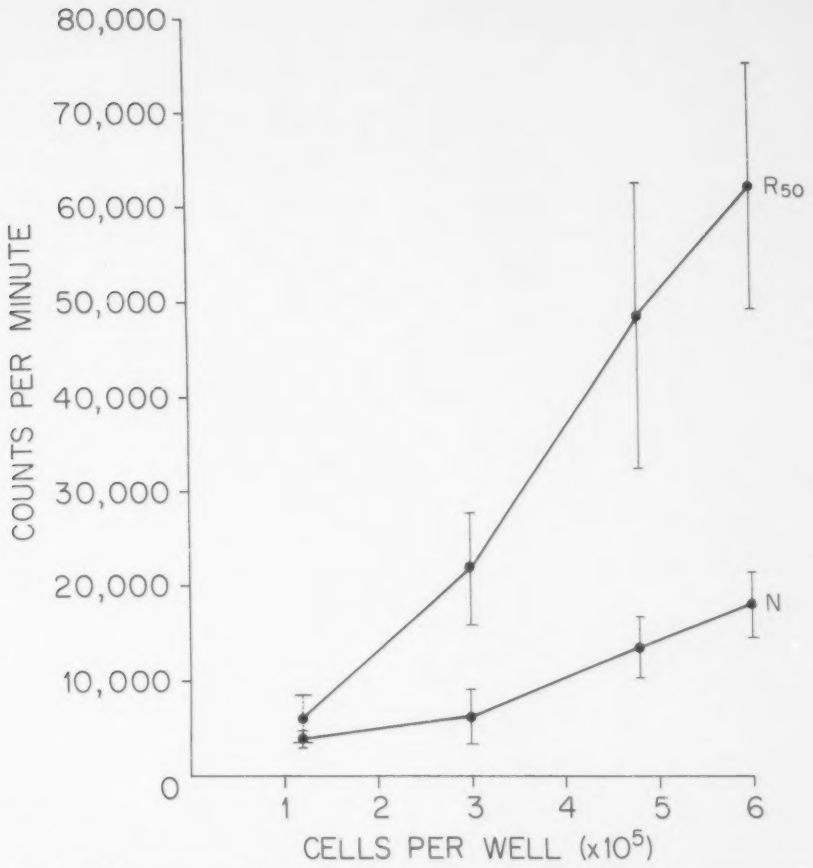

FIG. 3. Cell dosage study of spleen cell PHA reactivity in 13–14.5 month-old N and R_{50} mice. Values represent $\bar{X} \pm SE$ ^{3}H-TdR uptake for individual mice with background cpm (which ranged from 300 to 9000 and did not differ between N and R_{50}) subtracted. Four mice were tested per group.

vs. 86 ± 7 for N) and in lymph nodes (5 ± 1 × 10^6 cells per pool for R_{50} *vs.* 11 ± 2 for N). PHA-stimulated ^{3}H-TdR uptake (Table 4) was once again increased in spleen cells from R_{50} ($P < 0.001$); however, lymph node cell PHA responses from these same mice did not differ between the groups.

Levels of PHA-induced blast cells

Spleen cells from individual 5–6 month old N and R_{50} mice were stimulated with PHA and the percent of blast cells determined for individual spleens from which cells were also being cultured for assay of ^{3}H-TdR uptake. Results (Table 5) show that R_{50} mice had 3.0 fold fewer ($P < 0.001$) recoverable total cells per spleen but showed a 2.0 fold increase ($P < 0.02$) in PHA-stimulated ^{3}H-TdR uptake and a 1.7 fold increase ($P < 0.01$) in proportions of PHA-stimulated blast cells.

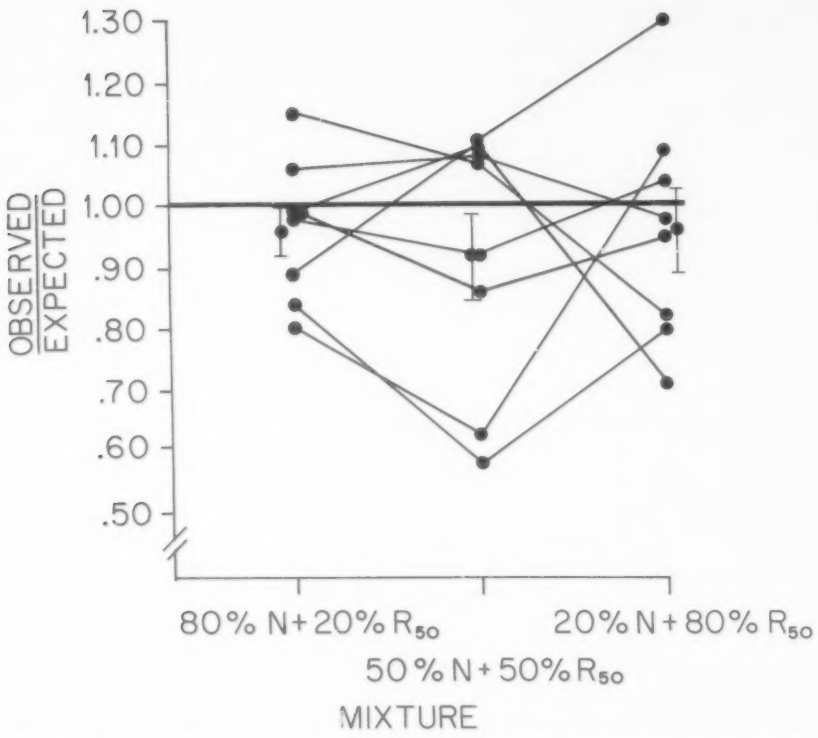

FIG. 4. PHA reactivity of mixed cultures of spleen cells from 13–14.5 month-old dietarily restricted and control mice. Each line connects points from a particular combination mixed in different proportions. Each vertical bar shows 2 *SE* of the group $\bar{X}$. Expected values were calculated on the basis of the response of 6×10^5 cells as described in Methods and Procedure. The ratio of observed: expected did not differ significantly from unity when compared by a one sample t test. Cells from the two N mice in each experiment were mixed as a control and found to respond as expected (data not presented). Observed cpm (with backgrounds subtracted) were: 80% N + 20% R_{50} = 26,845 ± 4297; 50% N + 50% R_{50} = 37,360 ±7474; 20% N + 80% R_{50} = 49,492 ± 5039.

Splenic T cell levels

These were determined in 5–5.5 month old N and R_{50} mice. Results appear in Table 6. R_{50} mice had a 3.2 fold decrease ($P < 0.001$) in total recoverable spleen cells accompanied by a 1.7 fold increase ($P < 0.01$) in the proportion of T cells as measured by cytotoxicity and a 2.0 fold increase ($P < 0.02$) in T cell levels as quantitated via immunofluorescence. Calculation of absolute splenic T-cell numbers for individual spleens indicate that spleens of N mice contain 1.4 fold (based on immunofluorescene) to 2.1 fold (based on cytotoxicity) more total T cells than are in R_{50} spleens.

DISCUSSION

In the present experiments, weaning-initiated dietary restriction resulted in: (a) a 2–4 fold decrease in the number of nucleated cells per spleen, (b) an increased splenic PHA

TABLE 4. INFLUENCE OF WEANING-INITIATED DIETARY RESTRICTION ON SPLENIC AND LYMPH NODE CELL PHA RESPONSES IN 12–13 MONTH OLD MICE[a]

Group	Splenic PHA	Lymph node PHA
N	22,253 ± 2056[b]	85,656 ± 4236
R_{50}	53,868 ± 4573	79,815 ± 8449

[a]Values represent $\bar{X} \pm SE$ for individual mice expressed as cpm ^{3}H-TdR corrected for unstimulated uptake (which ranged between 500–6000 for lymph node cells and 3000–9000 for spleen cells, and was not influenced by underfeeding). Seven mice per group were tested for both spleen and lymph node cell reactivity. Cultures were labeled at 24 hrs. and harvested at 48 hrs.

[b]Significantly different from R_{50} with $P < 0.001$.

proliferative response which could not be attributed to kinetic differences, (c) increased PHA responses by R_{50} spleen cells compared to N at both optimal and reduced cell densities, (d) equal PHA responses for lymph node cells from N and R_{50} mice, (e) an increase in spleen cell Con A reactivity in one of three experiments, (f) a sizeable influence on the mitogenic responses of the time since last feeding when restriction was done by intermittent feeding (Restricted mice not fed for 48 hours responded to a lesser extent to PHA and Con A than restricted mice not fed for 24 hours prior to sacrifice. Compared to N mice, fasted mice showed greater PHA reactivity, but marginally reduced Con A reactivity.), (g) no detectable synergy or suppression in mixed cultures of N and R_{50} spleen cells stimulated with PHA, (h) a 1.7 fold increase in proportions of PHA-stimulated splenic blast cells for R_{50} *vs.* N mice, and (i) a 1.7–2.0 fold increase in percent of splenic T cells for R_{50} *vs.* N mice but, because of the reduction in spleen cell numbers in R spleens, a 1.4–2.1 fold fall in absolute T-cell numbers per spleen.

These findings suggest that dietary restriction raises splenic PHA response capacity at least in part by increasing the proportion of PHA-responsive T cells. This view is strengthened by the observation that the increases in blast cell and T cell proportions in the restricted mice were of a magnitude similar to that of the increases in PHA-induced ^{3}H-TdR uptake. Additionally, higher responses were observed as limiting cell dilutions were approached. Likewise, if food restriction does not alter the time period required for initiation of PHA-induced mitogenesis, then the higher response by R_{40} cells at 0→24h

TABLE 5. INFLUENCE OF WEANING-INITIATED DIETARY RESTRICTION ON LEVELS OF PHA-STIMULATED BLAST CELLS

Group[a]	Total cells/spleen (x 10^6)	^{3}H-TdR uptake/culture[b]	% Blasts/culture[c]
N	96 ± 8[d]	17,948 ± 2962[e]	25 ± 3[f]
R_{50}	32 ± 6	35,843 ± 4723	42 ± 3

[a]Four mice tested per group. Mice 5–6 months old when tested.

[b]Values represent $\bar{X} \pm SE$ for 6×10^5 cells/culture for individual mice expressed as cpm ^{3}H-TdR corrected for unstimulated uptake (which ranged between 3000–7000 and did not differ between groups). Labeling was from 24→48 hours.

[c]Percent blasts determined microscopically at 48 hours. These values represent numbers with background levels subtracted. These background levels ranged from 8–19% and did not differ between groups (N = 12 ± 2 and R_{50} = 14 ± 2).

[d]Significantly different from R_{50} with $P < 0.001$.

[e]Significantly different from R_{50} with $P < 0.02$.

[f]Significantly different from R_{50} with $P < 0.01$.

TABLE 6. INFLUENCE OF WEANING-INITIATED FOOD RESTRICTION ON SPLENIC T CELL LEVELS[a]

Group[a]	Total cells/spleen (x 10^6)	% Cytotoxicity[b]	% Specific flourescence[c]
N	83 ± 6[d]	19 ± 3[e]	22 ± 7[f]
R_{50}	26 ± 4	32 ± 3	43 ± 3

[a]Values represent $\bar{X} \pm SE$ for $n = 9$ (cells/spleen), $n = 7$ (% cytotoxicity), $n = 5$ (fluorescence). Mice were 5-5.5 months old when tested.

[b]% cytotoxicity = % cells killed with anti-T cell serum + C′ minus % cells killed with C′ alone. Background cytotoxicity was not different between groups (N = 14 ± 2; R_{50} = 17 ± 2%) and ranged from 8-27%.

[c]% specific fluorescence = (% cells fluorescing in presence of monoclonal α-Thy 1.2 + F*) − (% fluorescing in presence of F* alone). Background fluorescence was increased in N mice compared to R_{50} (23 ± 4 vs. 16 ± 3%) but this was not statistically significant. Background fluorescence ranged from 15-35% for N and 10-25% for R_{50}.

[d]Significantly different from R_{50} with $P < 0.001$.

[e]Significantly different from R_{50} with $P < 0.01$.

[f]Significantly different from R_{50} with $P < 0.02$.

(Fig. 2) points toward the possibility that an increase in proportions of PHA-reactive cells would allow an earlier detection of response. The failure to observe either synergy or suppression in mixed cultures may be consistent with differences in frequency of responding units. The removal of adherent cells from N and R_{50} spleen cell suspensions did not differentially influence PHA responses (data not shown).

The increase in PHA responsive cells brought on by diet restriction contrasts with decreased proportions of PHA responsive cells in spleens from old mice (Hung *et al.*, 1975; Callard and Basten, 1977) and in peripheral blood from old humans (Inkeles *et al.*, 1977; Tice *et al.*, 1979). These two human investigations also found that PHA-stimulated peripheral blood lymphocytes from old people show longer mean cell cycle times and a lowered capacity for repeated divisions. The present data do not provide much solid information regarding division capacity; however, the tendency toward increasing PHA responses for restricted mice at 48→56h (Fig. 2) might be indicative of more cell divisions.

How might the present findings be explained in terms of T cell subtypes? Since PHA-responsive spleen T cells appear to be Lyt-$1^+2^+3^+$ and Con A-responsive T cells Lyt-$1^+2^-3^-$ (Hirst *et al.*, 1975; Nakayama *et al.*, 1980), increases brought on by underfeeding in splenic T cell proportions of Lyt-$1^+2^+3^+$ cells without changes in levels of Lyt-$1^+2^-3^-$ cells would correspond to our results. PHA-reactive peripheral T cells of mice were found to be cortisone-sensitive and of recent thymic origin, but unable to provide helper or suppresor activity (Callard and Basten, 1978). In young mice, Lyt-$1^+2^+3^+$ comprise about 25-35% of splenic and lymph node T cell populations, with the other 65-75% being Lyt-$1^+2^-3^-$ (Scollay *et al.*, 1978; Ledbetter *et al.*, 1980). In contrast, 90% of thymocytes were observed to express Lyt-$1^+2^+3^+$ with the remaining 10% being Lyt-$1^+2^-3^-$ and displaying cortisone-resistance and an increased expression of Lyt-1 (Mathieson *et al.*, 1979). Ledbetter and coworkers (1980) observed that Lyt-$1^+2^+3^+$ thymocytes and splenocytes tend to express less Lyt 1 and more Thy 1 than do Lyt-$1^+2^-3^-$ cells and this was viewed as indicative of a less mature profile for Lyt-$1^+2^+3^+$. The association in the majority of Lyt-$1^+2^-3^-$ thymocytes of cortisone-resistance, receptors for peanut agglutinin, and Ly-6^+ (London and Horton, 1980) also points toward increased phenotypic maturity for this subtype. Thymocytes with Lyt-$1^+2^+3^+$ or Lyt-$1^+2^-3^-$ phenotypes are found within all morphologic subgroups indica-

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tive of different stages of thymocyte maturation (Scollay and Weissman, 1980) leading these authors to suggest that two separate lines of thymocyte differentiation, as opposed to Lyt-$1^+2^+3^+$ serving as a common precursor, may exist. T cells emigrating from the thymus display the same Lyt balance (70% Lyt-$1^+2^-3^-$ plus 30% Lyt-$1^+2^+3^+$) as found in spleen and lymph nodes (Scollay *et al.*, 1978). A estimated 7% of thymocytes in 3.5–5.5 week-old mice emigrate daily and avoid the cell death which awaits about 95% of thymocytes (Scollay *et al.*, 1980). These authors also observed that 6 month-old mice show reduced thymus emigration rates compared to younger mice, and argue that this reduction may relate to age-related decreases in the cortex:medulla ratio of the thymus. We have reported that this ratio was increased by food restriction in 6 month-old B10C3F_1 mice (Weindruch and Suffin, 1980), which mice also showed elevated PHA and Con A reactivity (Weindruch *et al.*, 1979). The increased Con A response in these earlier studies suggests that Lyt-$1^+2^-3^-$ proportions can also be increased by food restriction in some mouse strains. The collected observations suggest that diet restriction might: (a) alter the proportions of Lyt-$1^+2^+3^+$ and Lyt-$1^+2^-3^-$ proliferating cells in the thymus, (b) influence which thymocytes die and/or the rates of thymocyte death, and (c) ameliorate age-related decreases in thymocyte emigration rates. Extrathymic T cell maturation (Stutman, 1978) could be influenced as well. In this regard, the lack of an effect of dietary restriction on lymph node PHA responses suggests that extrathymic mechanisms may be operative.

Relevant to the above possibilities is the observation that dietary restriction imposed on 12 month-old B10C3F_1 mice which until then had been fed normally led to increased spleen cell PHA and Con A ^{3}H-TdR uptake when studied at 16.5 months of age (Weindruch *et al.*, 1979), and that underfeeding started at 12, 17 or 22 months of age in C3B10RF_1 mice clearly raised PHA (but not Con A) responses when the mice were tested at various ages thereafter (Weindruch, *et al.*, 1982). These findings suggest that thymus structure may not critically influence the effect of food restriction on mitogenic responses since some effect is detectable in mice underfed beginning at either 1 month or 22 months of age.

Diverse diets and feeding protocols initiated on weanling mice of long-lived strains are reported to yield an increase in splenic lymphocyte mitogen responses. In our experiments underfeeding was accomplished by intermittent feeding of diets enriched in protein, vitamins and salts. Intermittent feeding of diets enriched in vitamins and salts but not in protein increased PHA and Con A responses of B10C3F_1 mice at several ages, the difference compared to controls being greatest in old mice (Cheney *et al.*, in preparation); however, these diets lowered responses to PHA, Con A, PWM and LPS in very young C57Bl/6J mice but increased reactivity in mice studied at or beyond mid-adulthood (Gerbase-DeLima *et al.*, 1975). PHA and Con A reactivity was increased in 3 month-old restricted C3H/Umc mice consuming non-protein enriched diets (Fernandes *et al.*, 1976). In all these studies mice were given a fixed amount of food. Mann (1978) carried out restriction by allowing C57Bl/6J mice free access to a standard diet for 24 hours on Mondays and Wednesdays and for 8 hours on Fridays. He observed lower splenic PHA and Con A responses for underfed mice after 1 month of feeding, but higher responses by 7 months of age. It was in this study that a fasting cycle effect was first reported. The present results with regard to the fasting cycle are confirmatory, but differ from Mann's in that our fasted mice showed: (a) less pronounced decreases in responses, and (b) a lower unstimulated ^{3}H-TdR uptake. Clearly, certain conflicting and confusing data in the litera-

ture might be explainable on the basis of experimental designs which ignored the acute nutritional status of an animal. Another type of underfeeding involves unrestricted feeding of low protein (usually 4–8%) diets. Rodents typically eat less of such diets and do not grow as fast or as large as controls. PHA responses have been reported to be increased in mice fed 6–8% casein diets for 1–3 months post weaning (Cooper *et al.*, 1974; Khorshidi and Mohagheghpour, 1979). We note that for these various diet strategies (which are often presumed to act via caloric restriction) fat intakes for restricted mice are less than for controls. This difference deserves recognition in view of the immunoinhibitory effects of dietary polyunsaturated fatty acids (Mertin and Hunt, 1976; Kollmorgen *et al.*, 1979). Yet another key difference among variously reported diet restriction studies involves the level of food intake for the control mice. In the present studies the "control" mice (Cohort N) were dietarily restricted relative to mice freely fed Purina Lab Chow.® Despite these complications, the studies by ourselves and others support the view that appropriate food restriction in the sense of "undernutrition" is quite different immunologically from "malnutrition", in that many T-dependent immunologic responses are more vigorous in the underfed. This enhanced or preserved vigor is associated with inhibition of late-life diseases and with increases in longevity.

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SUMMARY

Previous studies have shown that rodents dietarily restricted (but not malnourished) since weaning (3–4 weeks old) show prolonged maximum survivorship, inhibition of occurrence of late-life diseases and delayed onset of senescent changes in the immune system. Mitogen induced lymphocyte proliferation, known to decline with age, was examined using mice of a long-lived hybrid strain dietarily restricted at weaning. They were fed intermittently on diets enriched in protein, vitamins and salts and providing approximately 50–60% of the calories fed to control mice.

In six series of experiments dietarily restricted mice showed a 2–4 fold decrease in the number of nucleated cells per spleen and a 1.3–3.3 fold increase in phytohemagglutin (PHA)-induced tritiated thymidine uptake by spleen cells. However, diet restriction did not influence splenic responses to B cell mitogens (*E. coli* lipopolysaccharide and pokeweed mitogen). An increase in splenic concanavalin A (Con A) reactivity was observed in one of three experiments. The higher maximal PHA responses by restricted mice could not be attributed to kinetic differences or to mitogen concentrations. Spleen cells from restricted mice displayed higher PHA responses at both optimal and reduced cell densities. Lymph node cell yields were reduced by underfeeding but PHA responses were not altered by dietary restriction. The immediate fasting *vs.* feeding state of restricted mice affected both splenic PHA and Con A reactivity as restricted mice studied 48 hours from last feeding revealed lower PHA and Con A responses than were displayed by restricted mice fed 24 hours before being killed. Neither synergy nor suppression was detected in PHA-stimulated mixed cultures of control and restricted spleen cells. The increase in splenic PHA reactivity for restricted mice was associated with a 1.7 fold increase in the proportion of PHA stimulated blast cells (as judged microscopically) and a 1.7–2.0 fold increase in the proportion of splenic T cells (as shown by cytotoxicity and immunofluorescence measurements). This increase in T cell proportions in underfed spleens is not as great as the decrease in total spleen cells yielding a 1.4–2.1 fold fall in absolute T cell numbers in restricted spleens. These findings suggest that dietary restriction of a type

which may be described as "undernutrition without malnutrition" raises splenic PHA response capacity at least in part by increasing the proportion of PHA-responsive T cells.

Acknowledgements—We thank Drs. Susan R.S. Gottesman and Howard V. Raff for critically evaluating the manuscript, Melvinia Harris for technical help, and Helga Bradish and Laurie Miele for secretarial assistance. This study was supported by United States Public Health Service grant AG00424. RHW is supported by National Research Service postdoctoral fellowship CA-9030 from the National Cancer Institute.

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Experimental Gerontology, Vol. 17, pp. 65–69, 1982
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SKIN TEMPERATURE GRADIENT IN THE LOWER LIMBS OF OLD WOMEN

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(*Received* 1 *April* 1981)

INTRODUCTION

ISCHAEMIC LESIONS of the feet and legs are not uncommon among elderly patients. Such lesions as arterial ulceration or senile gangrene, for example, are responsible for a certain amount of morbidity and even mortality. Hence the state of the arterial circulation in the lower limbs can be of considerable clinical importance. A study of skin temperatures has therefore been undertaken to assess the gradient of fall between the groins and toes in old age. This investigation supplements research into the temperatures of the toes among elderly females undertaken previously (Howell 1979).

MATERIALS AND METHOD

The subjects of the present enquiry were 104 convalescent old women under the care of the writer at Queen's Hospital, Croydon. Their ages, as shown in Table 1, ranged from 61 to 100 years. Temperatures were taken with an electric thermometer, made by Light of Brighton, which had been tested to an accuracy of ±0.2°C. Readings were made in both groins, popliteal spaces, the front of both ankles, and the balls of the great toes. All measurements were checked by two observers. Room temperature was taken at the time of recording. The investigation took just under a year to complete.

RESULTS

Skin temperature readings in the left groins varied from 33.8°C to 37.8°C. This gave a range of 4.0° with a standard deviation of 0.77°C. The mean temperature was 35.8°C. In the right groins, the lowest reading was 32.4°C and the highest 37.8°C. Hence the range was 5.4°; but the mean, 35.8°C, was the same as on the left side. The standard deviation in this site was 0.82°. In the left popliteal space, the range of readings lay between 26.0°C and 37.2°C; this amounted to 11.2°. The mean was 32.1°C and the standard deviation 1.95°C. In the right popliteal space, the range of 10.2° lay between 27.4°C and 37.6°C.

Requests for reprints should go to Dr. Howell at 21 Abbotswood Road, London SW16 1AJ, England.

TABLE 1. AGE OF SUBJECTS

Age group	Number
61-70	11
71-80	40
81-90	41
91-100	12
Total	104

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The mean here was 33.0°C and the standard deviation 1.82°. As far as the front of the ankles were concerned, the range on the left side was from 21.2° to 36.4°C. This gave a span of 15.2°, a mean of 30.4°C and a standard deviation of 3.26°. On the right, the range of 14.0° lay between 22.4° and 36.4°C, with a mean of 30.2°C. The standard deviation at this site was 3.34°. When the toes were reached, the left great toe temperature varied from 20.0° to 37.2°C. The range was 17.2° and the standard deviation 4.80°. The right great toe registered between 20.2° and 36.6°C. This gave a span of 16.4° with a standard deviation of 4.54°. (Table 2.)

STATISTICAL CONSIDERATIONS

There was no statistical difference between the right and left sides at the various locations where temperature was measured. ($p > 0.25$) Hence it is possible to say that the average readings in the different sites showed a gradient of definite fall, as shown in Figure 1. Mean groin temperature was 35.8°C. Mean popliteal reading was 32.9°C. The mean figure for the ankles was 30.3°C, while the mean toe temperature was 27.0°C. Hence the fall in temperature from groin to popliteal space amounted to 2.9°. From the popliteal space to ankle the difference was 2.6°. Between ankle and toe, the fall came to 3.3°. The total fall between groin and toe amounted to 8.8°C.

The steady fall in temperature between the groins and the toes was accompanied by an increased variation in readings. Such a phenomenon might be due either to the age of the subject or to the temperature of the circumambient air. When these hypotheses were tested, it was found that the relationship between groin temperature and age showed a correlation coefficient of $r = -0.207$ which was significant at the 5% level ($0.05 > p > 0.01$) Regarding ankle temperature and age, the correlation coefficient was $r = -0.252$, significant at the 1% level. ($0.01 > p > 0.005$). When the relationship between room

TABLE 2. TEMPERATURES AT SELECTED SITES (°C)

Site	Maximum	Minimum	Range	Mean	Standard deviation
Rt Groin	37.8	32.4	5.4	35.8	0.82
Lt Groin	37.8	33.8	4.0	35.8	0.77
Rt Popliteal Space	37.6	27.4	10.2	33.0	1.87
Lt Popliteal Space	37.2	26.0	11.2	32.1	1.95
Rt Ankle	36.4	22.4	14.0	30.2	3.34
Lt Ankle	36.4	21.2	15.2	30.4	3.26
Rt Great Toe	36.6	20.2	16.4	27.2	4.54
Lt Great Toe	37.2	20.0	17.2	26.8	4.80

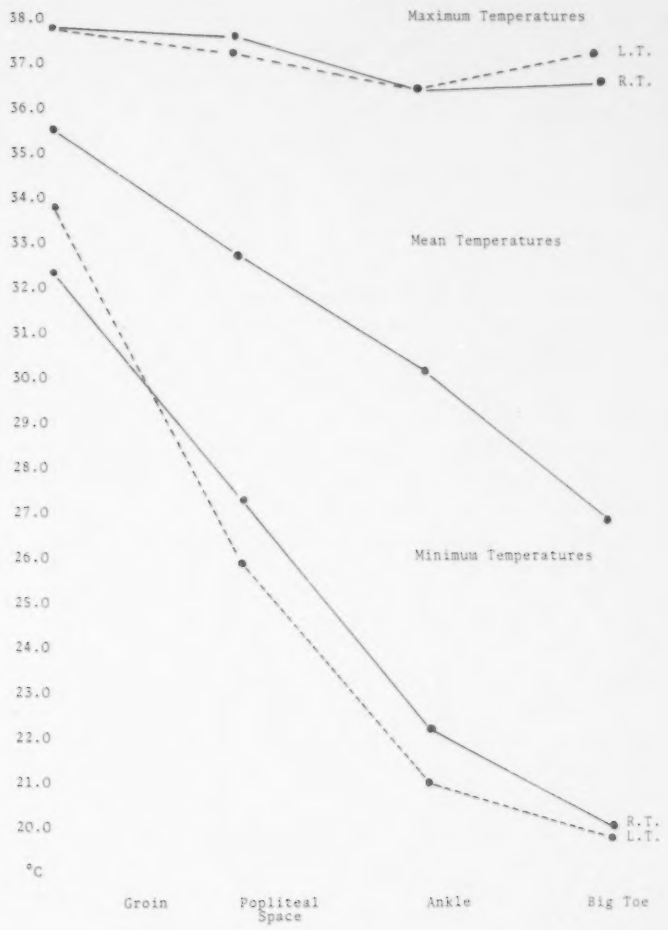

FIG. 1. Temperature gradient in lower limbs (°C).

temperature and groin temperature was studied, the correlation coefficient $r = 0.230$, which was significant at the 5% level. ($0.05 > p > 0.01$) As far as the ankle temperature was concerned, the correlation coefficient $r = 0.306$, which was significant at the 0.5 level. ($0.005 > p > 0.001$) Such findings indicate that room temperature is more significantly correlated to the lower limb figures than is the age of the subject; though all results are significant at the 5% level. As readings are made lower and lower down the limb, the correlations with both age and circumambient room temperature increase in significance. This would explain both the greater range and increased standard deviation as the arterial circulation progresses towards the extremities.

DISCUSSION

When the plotted readings are considered in Figure 1, several points of interest emerge. First of all, it seems that there is only a small drop in the maximum figures between groin

and toe. Then the maximum readings in the ankles are lower, in this series, than those for the great toe, which is unexpected. On the other hand, the *minimum* readings show a very different pattern. The fall between groin and popliteal fossa, between popliteal fossa and ankle, and from ankle to toe, is quite considerable. There is a difference of 7.8° between the left groin and the left popliteal space. From right groin to right popliteal space the difference is 5.0°C. The fall from left popliteal fossa to left ankle is 4.8°, while the difference on the right side is 5.0°. The gradient of minimum readings between ankle and great toe is not so steep; but, contrary to the level of maximum readings found, there is a drop and not a rise. This amounts to 1.2° on the left and 2.2° on the right. The mean of all temperature readings, however, makes almost a straight line on the graph, with its total drop of 8.8°C. between groin and great toe. These findings suggest that there are some old folk whose temperature gradient in the lower limb is quite small; while there are others whose toes are much colder than their groins. It is, presumably, the latter group who are in danger of having inadequate arterial circulation for the needs of their peripheral tissues.

It is of interest to compare the great toe temperatures in the present series of subjects with those of another group of 50 old women previously studied (Howell 1979). These subjects had a range of readings varying from 19.8 to 34.8°C on the right, and from 20.2 to 34.4°C on the left side. Histograms of the figures suggested that there might be a double population present, even among clinically similar old women; one group having a fully functioning homeostatic temperature control; the other failing to maintain a con-

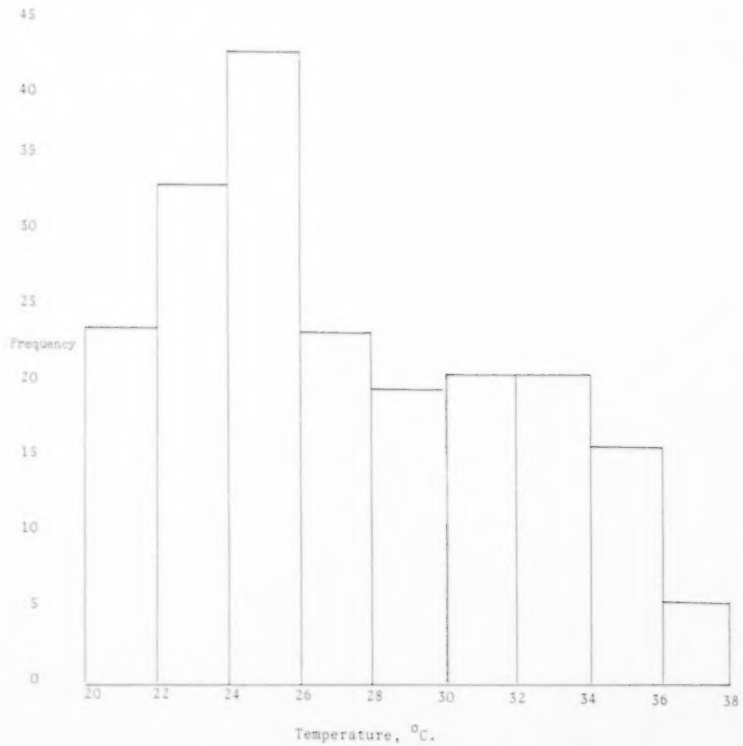

FIG. 2. Temperature frequencies—toes.

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stant regulated temperature. In the present series, while the range of readings was similar to that of the series, previously reported, there was no evidence of such a double population (Figure 2).

CONCLUSIONS

Among a group of 104 old women, there was a fall of mean skin temperature between groins and great toe amounting to 8.8°C.

The variations in temperature become greater from proximal to distal sites of readings. The range amounted to 16.4° for the right great toe and to 17.2° for the left great toe.

Such increased variations could be related both to the age of the subject and to circumambient temperature; the latter being more highly correlated.

Acknowledgements—My thanks are due to John Nicholas for his valuable help with statistics, and to the nursing staff of the outpatient department of Queen's Hospital, Croydon, for their assistance.

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Experimental Gerontology, Vol. 17, pp. 71–85, 1982
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AGING OF MEMBRANE TRANSPORT MECHANISMS IN THE CENTRAL NERVOUS SYSTEM

GABA Transport in Rat Cortical Synaptosomes

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(*Received* 20 *July* 1981)

INTRODUCTION

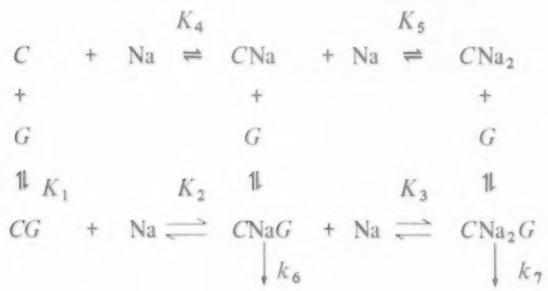
AN INEVITABLE consequence of aging is a decline in the ability to maintain homeostasis. The aging organism can no longer respond to stress with the efficiency characteristic of the young adult (Kohn, 1971; Gutmann and Hanzlikova, 1972; Diamond, 1978; Adelman, 1979; Masoro *et al.*, 1979). Since the nervous system plays such a major role in maintaining homeostasis, aging of the nervous system is likely to play a major role in the decline in homeostatic regulation (Franks, 1970; Gutmann, 1970; Sohal and Sharma, 1972; Hayflick, 1976; Diamond, 1978; Lytle and Altar, 1979).

What is the basis for the decline in nervous system regulation with age? Several studies point to the probability of an important role for synaptic function in aging: Electrophysiological correlates of synaptic function have been found to change with age (Frolkis *et al.*, 1972; Drechsler, 1978; Kelly, 1978; Landfield, McGaugh, and Lynch, 1978; Smith, 1979). Alterations of synthesizing and degradative enzymes, transmitter concentrations, transport capability, and post-synaptic receptors have also been found to occur (Kent, 1976a,b; Pradhan, 1980).

In order for a substance to function as a neural transmitter, there must be present a mechanism for removing the transmitter substance following stimulus-triggered release. Many neural transmitters, including γ-aminobutyric acid (GABA) and glutamate, are thought to be inactivated by a high affinity transport mechanism (Wheeler, 1980a). Thus aging changes in high affinity transport would be reflected in alterations in synaptic transmission and, as a consequence, in the regulatory ability of the nervous system. The probability that such changes in transport mechanisms play a role in aging of the nervous system, and hence in aging of the entire organism, has been amplified by previous studies in this laboratory of the aging of sodium dependent, high affinity glutamate transport (Wheeler, 1980b). Although the basic mechanism by which carrier, sodium, and

glutamate interact in the process of transport was found not to change with age, substantial quantitative changes were found to occur. The consequence of these changes is a reduced capacity for glutamate transport in the aged animals.

The hypothesis, then, is that altered membrane transport is a key factor in the aging process. The author has made detailed studies of the sodium-dependent, high affinity transport of GABA in synaptosomes from both cortex (Wheeler and Hollingsworth, 1979; Wheeler, 1980a; 1981a) and hypothalamus (Wheeler, 1980c) of 2-month old Long-Evans rats. Initial velocity of uptake of GABA was measured as a function of both GABA and sodium concentrations. Computer optimization techniques were then used to fit these data to the rate equation for each of several plausible models, and the minimal model giving best fit was identified. Although it appeared in the earlier studies that further simplification could be made, the following minimal model has now been shown to give minimal best fit to the data (Wheeler, 1981a):



C = carrier
G = GABA
K_1–K_5 = dissociation K's
k_6, k_7 = translocation constants
$K_6 = k_6[C]t$; $K_7 = k_7[C]t$

The present report extends such kinetic studies to 30-month old animals and compares the results to those found previously for 2-month old animals.

METHODS

Male Long-Evans rats 2–3 or 29–30 months old were used in this study. These rats were housed two per cage under conditions of constant temperature and controlled illumination, with *ad lib.* access to food and water. The diet was obtained from Wayne Feeds (Allied Mills, Inc., Libertyville, Ill.).

An extensive study of the suitability for membrane transport studies of the preparation and techniques used in the present study has been published (Wheeler, 1978). The methods described below evolved from this study.

Preparation of synaptosomes

Following decapitation with a guillotine, the brain was removed and placed on a filter paper moistened with Krebs-Henseleit solution (Lockwood, 1961) which was kept cold by placing on a petri dish filled with ice. The cortex was rapidly dissected free, weighed, and placed in 20 volumes of ice-cold 0.32 M sucrose in a 15-ml Potter-Elvehjem mortar fitted with a Teflon pestle. The clearance between mortar and pestle was 0.004–0.006 in. The homogenizer was placed in an ice water bath and the tissue homogenized for one minute, utilizing six 10-second strokes with a speed of 840 rpm. The homogenate was centrifuged for 10 minutes at 1085 × g at a temperature of 2°C to remove nuclei and cellular debris. The supernate was removed and centrifuged at 27,000 × g for 15 minutes to sediment the synaptosomes. The supernate from this procedure was decanted and discarded, and the synaptosomes were resuspended in 20 volumes of ice cold 0.32 M sucrose.

Measurement of uptake

The uptake of 14-C labelled γ-aminobutyric acid was measured by incubation of synaptosomes for one minute in a modified Krebs-Henseleit solution at 30°C. It has been shown in studies utilizing similar preparations that more than 95–98% of the GABA taken up is recoverable as GABA after 10 minutes of incubation (Martin and Smith, 1972; Ryan and Roskoski, 1977). Thus the amount of metabolism during one minute of in-

cubation would be negligible. 14-C labelled GABA (U.L., specific activity of 192 mCi/m-mol) was obtained from the New England Nuclear Corporation, Boston, MA, and was at least 98% pure. Non-labelled GABA was obtained from Sigma Chemical Company, St. Louis, MO. All incubations were carried out in a Dubnoff metabolic shaker. The modified Krebs-Henseleit used consisted of 127.2mM NaCl, 5.0mM KCl, 2.7mM$CaCl_2$, 1.3mM $MgSO_4$, 25mM Tris buffer, and 11.1mM glucose, at a pH of 7.4. The concentration of GABA in the final incubation medium was 5.0, 2.0, 1.0, 0.6667, or 0.5×10^{-4}M. For each sodium concentration used, a solution containing the highest amino acid concentration was prepared with a specific activity of 1×10^7 μCi/mol and serially diluted; thus a constant specific activity was maintained at all amino acid concentrations. Concentration of sodium in the incubation medium was varied by replacement of NaCl with equiosmolar Tris buffer; 0, 6, 12, 19, 27, 43, 69, 95 and 120.8mM sodium solutions were used with each of the above GABA concentrations. Incubation was begun by pipetting 0.2ml of the synaptosomal suspension (equivalent to 10mg of cortical tissue) into a scintillation vial containing 3.8 ml of incubation medium.

Following incubation, synaptosomes were separated from incubation medium by rapid filtration, utilizing a Millipore 30-place manifold and Millipore filters (HAWP02500). The synaptosomes were then washed with a 5-ml aliquot of Krebs-Henseleit ([Na] = 0) at 30°C. Previous studies indicate that such a wash procedure results in little loss of label from within the synaptosomes (Wheeler, 1978). Following completion of all incubations, each filter was removed, the bottom side blotted on filter paper, placed in a scintillation vial, and one ml distilled water added. After sitting for one hour, scintillation fluid was added; 24 hours later, the vials were counted for radioactivity in a Packard Model 3320 liquid scintillation spectrometer until the counting error was less than 1% at the 95% confidence level. Disintegrations per minute were calculated from counts per minute by use of an external standard to determine counting efficiency.

Initial velocity for each [*G*] and 0, 19, 27, 43, or 120.8mM [Na] was measured in each animal in one group of animals; in a second group, initial velocity for each [*G*] and 6, 12, 69, 95, or 120.8mM [Na] was measured. The 120.8mM [Na] data were then used to normalize the data between the two groups.

Blanks

In order to correct for filter retention of label when incubation medium is poured through them, blanks were run with each experimental solution. A 3.8 ml aliquot of incubation medium was added to a well of the filtration manifold, followed by the usual wash procedure. Filters were then treated as described above. Twenty-three repetitions were done with each GABA concentration. The amount of GABA trapped by the filters was computed and plotted as a function of GABA concentration. The data were then fitted to a straight line by linear regression; predicted values from this fit were determined for each GABA concentration and used for blank values. Initial velocity measurements were corrected by subtracting the blank value.

Intra-synaptosomal water content

Distributions of 14-C-polyethylene glycol (PEG) and 3-H-D-galactose were utilized to compute synaptosomal water content. Synaptosomes were prepared as usual. Following removal of the supernate from the first centrifugation, sufficient PEG and D-galactose were added to bring the concentration of each to 10^{-4}M, including 0.05 μCi/ml PEG and 0.2 μCi/ml D-galactose. After standing for two hours in an ice bath, synaptosomes were pelleted as usual. The supernate was sampled and counted for both carbon-14 and tritium. The remaining supernate was then removed and 5 ml distilled water added to the pellet, vortexed, and allowed to stand for 1 h. The preparation was centrifuged again at 27,000g for 15 min, and the supernate was sampled and counted for both carbon-14 and tritium. After correcting the counts for crossover, intra-synaptosomal volume was computed as the difference between the D-galactose and PEG volumes of distribution. Mean values for the 2-month group were 1.433 ± 0.03 (SEM) μl/10 mg tissue weight compared to 1.467 ± 0.04 for the aged group. Thus there is no significant difference between the two groups with regard to synaptosomal volume; therefore, the data were not corrected for water content, but were expressed per 10 mg tissue wet weight.

Computer optimization

In order to fit the data to the velocity equations for the various models, a parameter optimization approach was used to minimize the following function:

$$J = \sum_{i=1}^{n} \left(\frac{V_{\text{model}} - V_{\text{data}}}{V_{\text{data}}} \right)^2$$

J, the objective function, is the sum of the squared fractional errors between the velocities of uptake predicted by the models and those observed experimentally. The parameter optimization algorithm used in this research was

based on the Flexible Polyhedron approach of Nelder and Mead (1965). The algorithm was coded in BASIC and run on a Challenger II microcomputer (Ohio Scientific Instruments) fitted with 48K bytes of memory and a dual drive floppy disk.

To use the BASIC program, the appropriate model equation and velocity data were first added to the optimization portion of the program. Initial guesses for all kinetic constants were then entered, and program operation was begun. After the algorithm returned the optimal parameters, the program was restarted several times to insure that the minimum possible error had been achieved. Finally, the program printed the various data velocities paired with the corresponding values predicted by the equations.

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RESULTS

Comparison of initial velocities

Table 1 shows a comparison of initial velocities of uptake for 2- and 30-month animals. With one exception ([Na] = 6mM; $[G] = 5 \times 10^{-6}$M), GABA uptake has fallen in the aged animals at any combination of [Na] and [G]. As a percentage, uptake in the aged group ranges from 78% to 106% of that for the control group. When velocities are compared at each combination of [Na] and [G], most of the differences are highly significant except for the lowest [Na] used (6mM). At this low [Na], rate of uptake is small (Fig. 1), and although uptake is lower for 30-month animals for all [G]'s used except for 5×10^{-6}M, the differences between 2- and 30-month animals are not significant.

Best fit model

The data were fitted to the model found to give minimal best fit to the data for 2-month animals:

TABLE 1. COMPARISON OF INITIAL VELOCITIES
(v[30]/v[2]) × 100

$[G] \times 10^6$,M	0.5	0.6̄	1.0	2.0	5.0
[Na], mM					
6	94.59	83.76	91.10	95.48	106.19
	(.7–.6)	(.2–.1)	(.4–.3)	(.6–.5)	(.5–.4)
12	88.12	87.82	91.29	85.54	90.62
	(.1–.05)	(.02–.01)	(.05–.02)	(.01–.001)	(.2–.1)
19	77.81	83.85	85.59	84.40	90.82
	(.01–.001)	(.01–.001)	(.05–.02)	(.02–.01)	(.2–.1)
27	87.39	87.87	86.13	93.14	93.47
	(.02–.01)	(.02–.01)	(.01–.001)	(.2–.1)	(.3–.2)
43	87.69	90.31	86.26	89.68	91.50
	(.01–.001)	(.05–.02)	(.01–.001)	(.02–.01)	(.2–.1)
69	86.02	86.90	85.13	87.22	87.09
	(.01–.001)	(.01–.001)	(.01–.001)	(.05–.02)	(.02–.01)
95	86.48	88.0	89.76	91.92	90.19
	(.01–.001)	(.05–.02)	(.1–.05)	(.2–.1)	(.1–.05)
120.8	88.80	89.15	88.95	88.12	87.96
	(.01–.001)	(.01–.001)	(.01–.001)	(.01–.001)	(.01–.001)

P values are given in parentheses. Uptake at zero [Na] is close to zero for both groups, and is therefore not included. One-minute uptake was measured as an estimation of initial velocity.

$$
\begin{array}{ccccccc}
 & & & K_4 & & K_5 & \\
C & + & \mathrm{Na} & \rightleftharpoons\ C\mathrm{Na} & + & \rightleftharpoons\ C\mathrm{Na}_2 & C = \text{carrier} \\
+ & & & + & & + & G = \text{GABA} \\
G & & & G & & G & K_1\text{-}K_5 = \text{dissociation } K\text{'s} \\
\Updownarrow K_1 & & & K_2\ \Updownarrow & & K_3\ \Updownarrow & k_6, k_7 = \text{translocation } K\text{'s} \\
CG & + & \mathrm{Na} & \rightleftharpoons\ C\mathrm{Na}G & + \mathrm{Na} \rightleftharpoons & C\mathrm{Na}_2G & K_6 = k_6\,[C]\,t \\
 & & & \downarrow k_6 & & \downarrow k_7 & K_7 = k_7\,[C]\,t
\end{array}
$$

$$
v = \frac{\dfrac{K_6[\mathrm{Na}][G]}{K_1K_2} + \dfrac{K_7[\mathrm{Na}]^2[G]}{K_1K_2K_3}}{1 + \dfrac{[G]}{K_1} + \dfrac{[\mathrm{Na}][G]}{K_1K_2} + \dfrac{[\mathrm{Na}]^2[G]}{K_1K_2K_3} + \dfrac{[\mathrm{Na}]}{K_4} + \dfrac{[\mathrm{Na}]^2}{K_4K_5}} \tag{1}
$$

The fit of the experimental data to the model is excellent; the average error per data point (exclusive of the [Na] = 0 data) is only 1.48% compared to 3.05% for the 2-month data. Thus aging has not changed the basic mechanism by which sodium and GABA interact with the carrier in the process of uptake. However, the best fit constants which quantitate the model have changed with age (Table 2). As a result of the changes in these constants, there are significant quantitative differences in the transport mechanism between control and aged animals. The general nature of the consequences of the differences in kinetic constants is reflected in Table 1 as a decreased velocity of uptake in aged animals at any given [Na] and [*G*]. Further consequences of the differences in kinetic constants will be shown below.

The rate equation for the minimal best fit model

The rate equation can be rearranged as follows:

$$
v = \frac{\left\{\dfrac{K_3K_6[\mathrm{Na}] + K_7[\mathrm{Na}]^2}{K_2K_3 + K_3[\mathrm{Na}] + [\mathrm{Na}]^2}\right\}[G]}{\left\{\dfrac{K_1K_2K_3K_4K_5 + K_1K_2K_3K_5[\mathrm{Na}] + K_1K_2K_3[\mathrm{Na}]^2}{K_2K_3K_4K_5 + K_3K_4K_5[\mathrm{Na}] + K_4K_5[\mathrm{Na}]^2}\right\} + [G]} = \frac{V_a[G]}{Kt + [G]} \tag{2}
$$

We can substitute the constants obtained from the optimization process into the rate equation and compute predicted velocity as a function of [Na] and [*G*] (Figure 1). As expected, the shapes of the curves are similar in all respects for both 2- and 30-month data. At all combinations of [Na] and [*G*], except 6mM [Na] and 5×10^{-6}M [*G*], predicted uptake is less for the aged group. The above rate equation also enables us to mathematically define a number of parameters which are useful in determining what aspects of transport have changed with age.

V∞, *initial velocity of uptake at infinite [Na] and* [G]

Hypothetical maximal velocity would occur when both [Na] and [*G*] were infinite. Under these conditions, the rate equation reduces to $v = K_7$. K_7 for control animals is 1.2071 n-mol/10 mg-min compared to 0.9677 for aged animals (Table 2). The ratio of

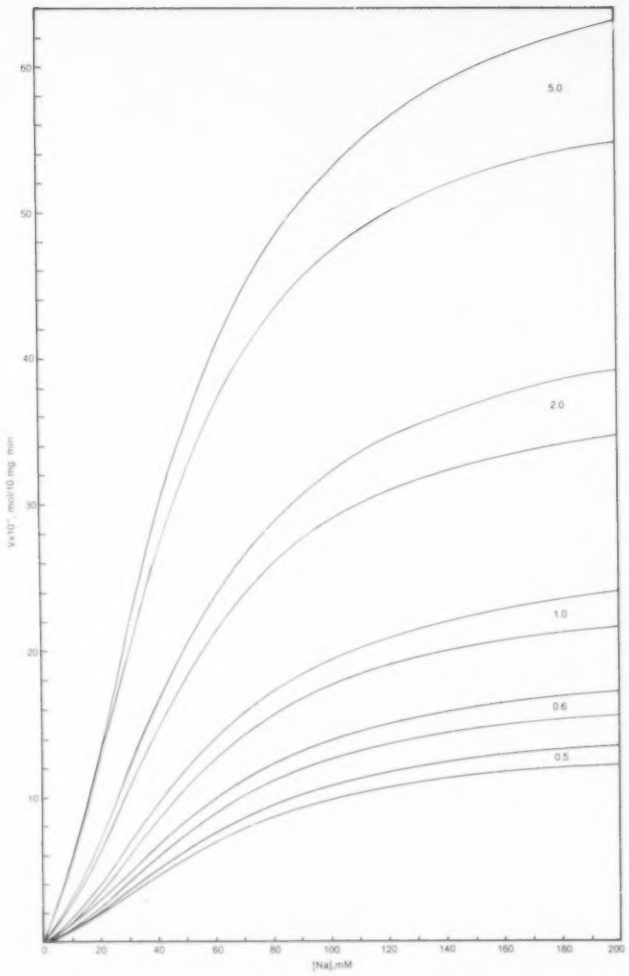

FIG. 1. Velocity of uptake (computed fit) as a function of sodium concentration for each of the GABA concentrations used. GABA concentration (μM) is given between each pair of curves; 30-month curves are the lower of the two curves in each case.

$V\infty(30)/V\infty(2) = 0.802$; thus under conditions of high [Na] and high [G], GABA transport capacity has declined approximately 20% in the aged animals.

GABA concentration which gives $V\infty/2$

If only the [Na] is infinite, the rate equation reduces to

$$v = K_7[G]/((K_1K_2K_3/(K_4K_5)) + [G]) = Jm \qquad (3)$$

Uptake at infinite [Na] and a given [G] is defined as Jm. If $K_1K_2K_3/(K_4K_5) = [G]$, then

TABLE 2. CONSTANTS GIVING BEST FIT

K	2-Month	30-Month
1	1.3781×10^{-5}	1.8704×10^{-5}
2	2.9118×10^{-2}	3.1940×10^{-2}
3	2.9900×10^{-2}	2.5601×10^{-2}
4	1.9112×10^{-1}	2.1690
5	1.8585×10^{-2}	2.2273×10^{-3}
6	2.4775×10^{-1}	4.4285×10^{-1}
7	1.2071	9.6773×10^{-1}

$K_6 = k_6[C]t$; $K_7 = k_7[C]t$; units for K_6 and K_7 are n-mol/10mg tissue minute.

$$V = \frac{K_7[G]}{2[G]} = \frac{K_7}{2}$$

and the velocity of uptake would be half that at infinite $[G]$. Thus the GABA concentration which gives $K_7/2$ is a measure of the reciprocal of the apparent carrier affinity for GABA under conditions of high [Na]. This $[G]$ for 2-month animals is 3.3779×10^{-6}M, compared to 3.1658×10^{-6}M for 30-month animals. Thus $K_7/2$ occurs at only a slightly lower $[G]$ for aged animals.

V_a, *apparent maximal rate of uptake*

V_a, the rate of uptake at infinite $[G]$, is a measure of the GABA transport capability at high $[G]$ and is dependent on the sodium concentration.

$$V_a = \frac{K_3K_6[\mathrm{Na}] + K_7[\mathrm{Na}]^2}{K_2K_3 + K_3[\mathrm{Na}] + [\mathrm{Na}]^2} \tag{4}$$

At infinite [Na], equation 4 reduces to $V_a = K_7$; at [Na] = 0, the equation reduces to 0. As discussed above, K_7 is 20% lower in aged animals. V_a's for 2- and 30-month animals, respectively, are (n-mol/10 mg-min) 0.0809–0.1021(1.26), 0.1913–0.2170(1.13), 0.3203–0.3392(1.06), 0.4487–0.4520(1.01), 0.6368–0.6043(0.95), 0.8133–0.7338(0.90), 0.9106–0.7993(0.88), 0.9704–0.8372(0.86), and 1.0016–0.8564(0.86) at [Na]'s of 6, 12, 19, 27, 43, 69, 95, 120.8, and 140mM, respectively. Ratio of $V_a(30)/V_a(2)$ is given in parentheses. Plots of V_a as a function of [Na] are shown in Figure 2; V_a is sigmoidally related to [Na] and approaches K_7 at infinite [Na]. Below 30mM [Na], V_a is greater for the aged group; above 30mM, V_a is less for this group, and the difference between the two groups increases as [Na] is increased. At 140mM [Na], transport capacity under conditions of high $[G]$ for the control group is 17% greater than that for the aged group.

Apparent Michaelis-Menten constant

Kt, the GABA concentration giving a velocity of uptake equal to $V_a/2$, is defined by the minimal best fit model as follows:

$$Kt = \frac{K_1K_2K_3K_4K_5 + K_1K_2K_3K_5[\mathrm{Na}] + K_1K_2K_3[\mathrm{Na}]^2}{K_2K_3K_4K_5 + K_3K_4K_5[\mathrm{Na}] + K_4K_5[\mathrm{Na}]^2} \quad (5)$$

At [Na] = 0, $Kt = K_1 = 1.3781 \times 10^{-5}$M for control animals compared to 1.8704×10^{-5}M for aged animals. Thus at low [Na], the apparent carrier affinity for GABA in control animals is 1.36 times that in aged animals. As [Na] approaches infinity, Kt approaches $K_1K_2K_3/(K_4K_5)$. As pointed out above, $K_1K_2K_3/(K_4K_5) = 3.3779 \times 10^{-6}$M for control animals compared to 3.1658×10^{-6}M for aged animals (ratio of 1.07). Thus under conditions of very high [Na], apparent carrier affinity is slightly greater for aged animals. At [Na]'s of 6, 12, 19, 27, 43, 69, 95, 120.8, and 140mM, Kt's for control and aged animals (μM), respectively, are (ratios are in parentheses) 11.51–15.34(1.33), 9.64–12.48(1.28), 8.01–9.95(1.24), 6.71–7.95(1.18), 5.22–5.69(1.09), 4.21–4.20(0.998), 3.80–3.63(0.96), 3.61–3.37(0.93), and 3.53–3.26(0.92). Kt as a function of [Na] is shown in Figure 3. Kt declines rapidly with increases in [Na] and approaches $K_1K_2K_3/(K_4K_5)$ as a limiting value. Although Kt is much higher for aged animals at low [Na]'s, the functions cross at about 70 mM [Na], and above this concentration, Kt is slightly lower for aged

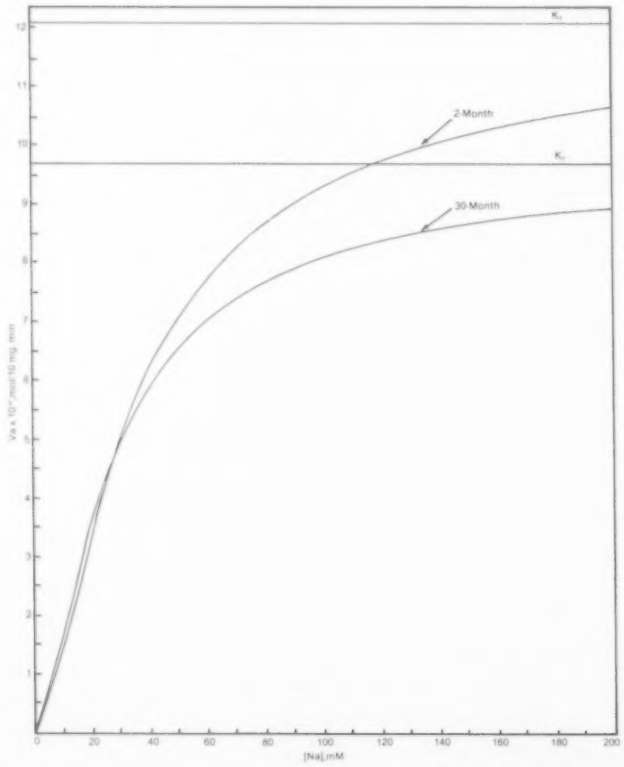

FIG. 2. *Va*, the apparent maximal velocity of uptake, as a function of sodium concentration. Equation 4 was used to compute *Va*.

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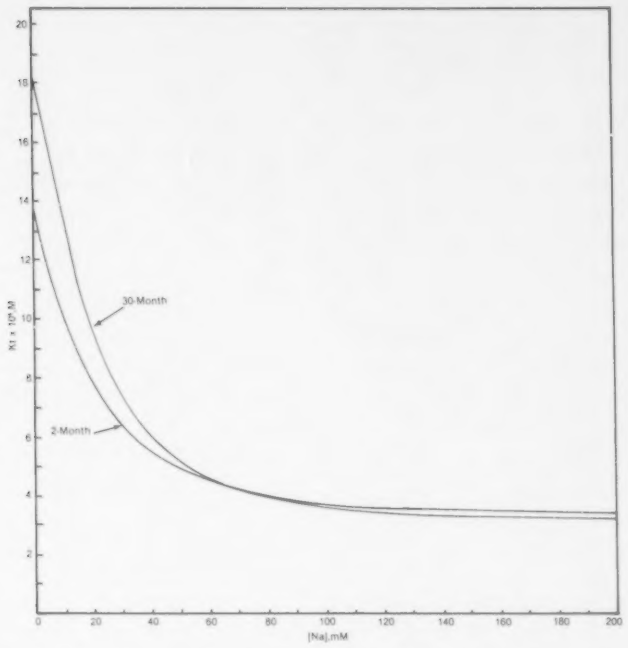

FIG. 3. K_t, the GABA concentration which gives a velocity of uptake equal to $Va/2$, as a function of sodium concentration. Equation 5 was used to compute K_t.

animals over a wide range of [Na]'s. Thus in the physiological range of [Na], the apparent carrier affinity for GABA is slightly greater for aged animals. Optimal Kt is given by $K_1K_2K_3/(K_4K_5)$. At 140 mM, $Kt/(K_1K_2K_3/(K_4K_5)) = 1.04$ for control animals, compared to 1.03 for aged animals. Thus at physiological [Na], apparent carrier affinity is very close to the optimal value for both control and aged animals.

Jm, *rate of uptake at infinite [Na] and a given* [G]

Jm as a function of [*G*] is given by equation 3:

$$Jm = \frac{K_7[G]}{K_1K_2K_3/(K_4K_5) + [G]}$$

Thus *Jm* is hyperbolically related to the GABA concentration and approaches K_7 as a limiting value (Fig. 4). *Jm* has declined substantially with age at all GABA concentrations. *Jm*'s for control and aged animals, respectively, are 1.56–1.32, 1.99–1.68, 2.76–2.32, 4.49–3.75, 7.20–5.93, 9.02–7.35, 11.31–9.10, and 11.68–9.38 $\times$ 10^{-10} mol/10mg min at [*G*]'s of 0.5, 0.$\bar{6}$, 1.0, 2.0, 5.0, 10, 50, and 100 $\times$ 10^{-6}M. The ratio of $Jm(30)/Jm(2)$ varies from 0.85 at 0.5 $\times$ 10^{-6}M to 0.80 at 100 $\times$ 10^{-6}M. Thus the transport capability of the carrier under conditions high [Na] has declined by about 15–20% with age.

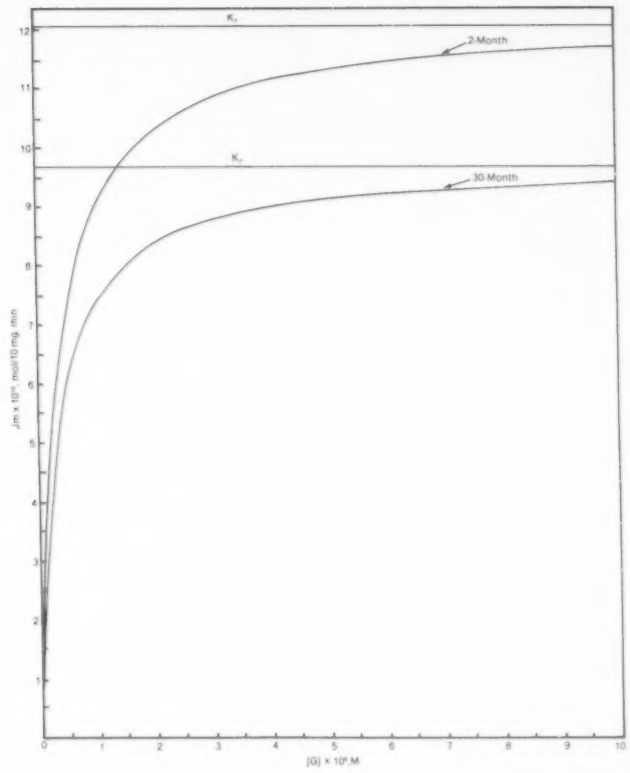

FIG. 4. J_m, rate of uptake at infinite [Na], as a function of GABA concentration. Equation 3 was used to compute J_m.

$\varkappa_{Na}$, *the [Na] giving a velocity of uptake equal to* Jm/2

$\varkappa_{Na}$ is a measure of the apparent carrier affinity for sodium at a given [G] and can be estimated graphically from Figure 1, or $\varkappa_{Na}$ can be found by solving the rate equation for [Na] when $v = Jm/2$. $\varkappa_{Na}$'s (mM) for control and aged animals, respectively, are (ratios are in parentheses) 61.1–57.1(0.93), 60.0–56.3(0.94), 58.8–54.9(0.93), 56.1–51.5(0.92), 51.2–45.4(0.89), 47.4–40.5(0.85), 41.8–32.9(0.79), 40.8–31.4(0.77), at [G]'s of 0.5, 0.$\overline{6}$, 1.0, 2.0, 5,0, 10.0, 50.0, and 100.0 $\times$ 10^{-6}M, respectively. Although the difference is minimal for the lower [G]s, $\varkappa_{Na}$ is lower for aged animals over the entire range of [G]'s examined (Figure 5). Thus the apparent carrier affinity for sodium has increased with age. For both control and aged animals, $\varkappa_{Na}$ is much less than normal [Na] for all [G]'s; therefore, the carriers function at a rate greater than $Jm/2$ in all cases. Figure 5 shows a plot of $\varkappa_{Na}$ as a function of -log [G]; $\varkappa_{Na}$ declines as -log [G] declines along an S-shaped curve.

Contribution of each pathway to total uptake

There are two possible pathways for uptake—as $CNaG$ or as CNa_2G. We can evaluate the contribution of each pathway to total uptake by separating the rate equation into its two components:

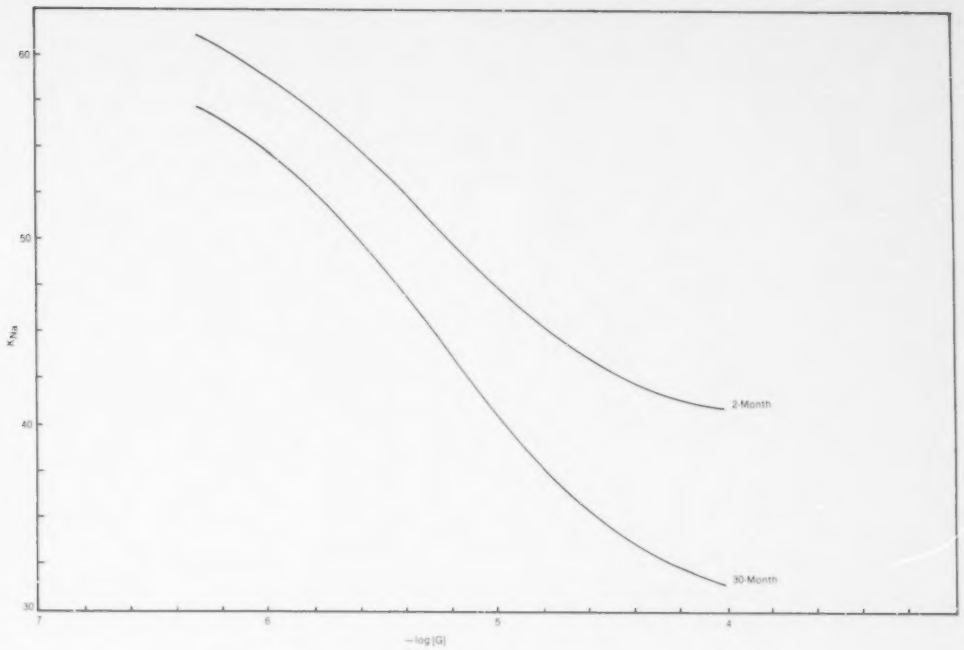

FIG. 5. x_{Na}, the sodium concentration giving a velocity of uptake equal to $J_m/2$, as a function of GABA concentrations.

$$Vt = V_1 + V_2$$

$$Vt = \frac{\dfrac{K_6[\mathrm{Na}][G]}{K_1K_2}}{D} + \frac{\dfrac{K_7[\mathrm{Na}]^2[G]}{K_1K_2K_3}}{D} \tag{6}$$

D = denominator of the rate equation. Table 3 shows the percentage of uptake by each pathway as a function of [Na]. At any [Na], the percentage by each pathway is invariant with GABA concentration; however, the relative uptakes by the two pathways change rapidly with sodium concentration. For both groups, the relative contribution by pathway 1 ($CNaG$) declines with [Na], while that of pathway 2 (CNa_2G) increases with [Na]. At 6mM [Na], 50.6 and 66.1% of total uptake takes place by pathway 1, while at 140mM [Na], 95.8 and 92.3% takes place by pathway 2 for the 2- and 30-month groups, respectively. If we divide the expression for V_2 by that for V_1, we obtain the following:

$$\frac{V_2}{V_1} = \frac{K_7[\mathrm{Na}]}{K_3K_6} \tag{7}$$

Figure 6 shows a plot of V_2/V_1 as a function of [Na]. At any given [Na], uptake by pathway 2 is relatively more important for the control group than for the 30-month group. If $V_2 = V_1$, then

TABLE 3. PERCENTAGE OF TOTAL UPTAKE BY EACH OF THE TWO PATHWAYS

	P_1		P_2	
[Na], mM	2M	30M	2M	30M
6	50.6	66.1	49.4	33.9
12	33.8	49.4	66.2	50.6
19	24.4	38.1	75.6	61.9
27	18.5	30.3	81.5	69.7
43	12.5	21.4	87.5	78.6
69	8.2	14.5	91.8	85.5
95	6.1	11.0	93.9	89.0
120.8	4.8	8.8	95.2	91.2
140	4.2	7.7	95.8	92.3
200	3.0	5.5	97.0	94.5

P_1 = $CNaG$ pathway; P_2 = CNa_2G pathway.

$$[Na] = K_3K_6/K_7 \tag{8}$$

A [Na] of 6.1mM gives equal uptake by the two pathways for the 2-month animals, while a [Na] of 11.7mM gives equal uptake for the aged animals.

DISCUSSION

The data for the 30-month animals were found to give an excellent fit to the minimal best fit model found previously for 2-month animals. Thus the fundamental mechanism by which carrier, sodium, and GABA interact in the process of uptake appears not to have changed with age.

However, as indicated by the best fit constants, quantitative changes have taken place in the carrier mechanism with age (Table 2). Constants K_1-K_5 are dissociation constants; the observed changes in these constants are indicative of changes in the balance of the several carrier species with age. Constant $K_6 = k_6[C]t$ and $K_7 = k_7[C]t$. Changes in these constants reflect changes in translocation constants (k_6, k_7), total carrier concentration, or both. Constant K_6, which is the theoretical maximum rate of uptake as $CNaG$, has increased with age. This is reflected in a greater contribution of the $CNaG$ pathway to total uptake in the aged group. Constant K_7, which is the maximal rate of uptake as CNa_2G, has declined with age. Thus either the rate constant for translocation (k_7) or the total carrier concentration, or both, has declined with age.

The differences in kinetic constants between the two groups result in differences in the computed parameters which describe uptake: (1) At any combination of [Na] and [G], uptake is less in the aged animals, the percentage difference between the two groups depending on the particular [Na] and [G]. (2) $V\infty$, uptake at infinite [Na] and [G], has declined with age by 20%. (3) At all except [Na]'s less than 30 mM, Va, the apparent maximal velocity of uptake, declined with age. At 140mM [Na], Va declined by 14%. (4) Kt, the [G] giving a velocity equal to $Va/2$, also changed with age. At low [Na]'s (below 70mM), Kt increased with age, while at higher [Na]'s, Kt declined slightly with age. At 140mM [Na], Kt declined by 8% in the aged group; thus the apparent carrier affinity at physiological [Na] increased with age. (5) Jm, the rate of uptake at a given [G] and high

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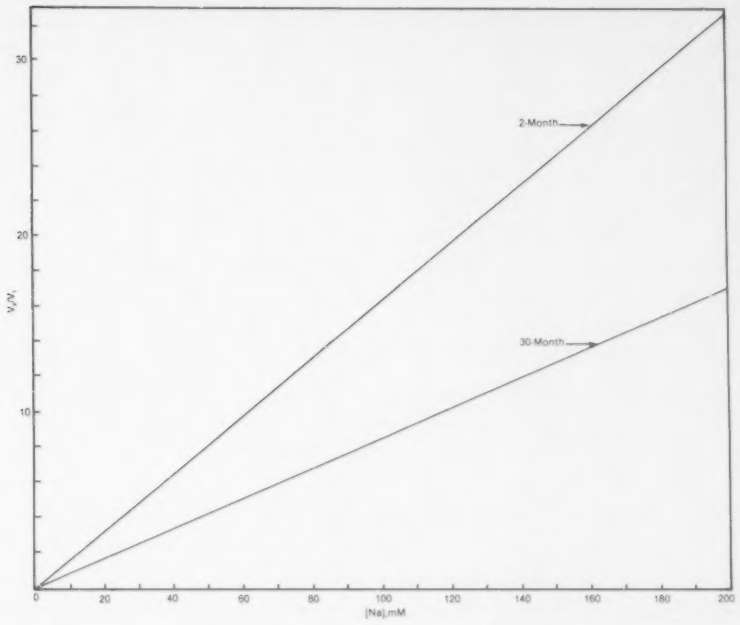

FIG. 6. Ratio of uptake by the CNa_2G pathway to that by the $CNaG$ pathway as a function of sodium concentration. Equation 7 was used to calculate V_2/V_1.

[Na], declined by about 15–20% with age. (6) $\varkappa_{Na}$, the [Na] which gives $Jm/2$, declined with age for all [G]'s. Thus the apparent carrier affinity for sodium increased with age. (7) The rate constants for translocation have changed with age. (8) Total carrier concentration may have changed with age. (9) The relative contribution by pathway 1 ($CNaG$) increased with age.

Table 4 shows a comparison of the present results with those of a previous study of the aging of high affinity glutamate transport in a similar preparation (Wheeler, 1980b). Qualitatively, many of the changes which were found to occur in the glutamate study are also found in the present study of GABA transport. However, the magnitudes of the changes differ somewhat in the two studies.

TABLE 4. COMPARATIVE EFFECTS OF AGING ON TRANSPORT MECHANISMS FOR GABA AND GLUTAMATE

	GABA	Glutamate
1. Initial velocity	Declined by 0–22%	Declined by 13–27%
2. Best fit model	Did not change	Did not change
3. $V\infty$	Declined by 20%	Declined by 28%
4. [S] giving $V\infty/2$	Declined by 6%	Declined by 6%
5. Va, [Na] = 140 mM	Declined by 14%	Declined by 28%
6. Kt, [Na] = 140 mM	Declined by 8%	Declined by 10%
7. Jm	Declined by 15–20%	Declined by 28–33%
8. $\varkappa$Na	Declined by 7–23%	Declined by ~10%
9. V_2/V_1	Declined	Did not change

In a study of the aging of synaptosomal transport in mouse brain, Jonec and Finch (1975) found uptake of dopamine by hypothalamic or striatal synaptosomes had declined by as much as 30% when 28-month animals were compared to 8-month animals. We have found alterations with age in the mechanisms for both glutamic acid transport and GABA transport. Thus aging of transport mechanisms in the nervous system may be a widespread phenomenon. Membrane transport processes are crucial to neuronal function, not only in the process of synaptic transmission, but also in maintaining cellular homeostasis. In a study of 2-deoxy-D-glucose (2-DG) uptake, no changes were found in transport capability with age (Wheeler, 1981b). 2-DG uptake is *via* a low affinity mechanism that is not directly involved in transmitter function, while those mechanisms which have been found to change with age are high affinity mechanisms which are thought to play a role in transmitter function. Thus high affinity mechanisms, necessary for synaptic function, may be more susceptible to the effects of age than are those low affinity mechanisms, which are necessary for maintaining cellular homeostasis.

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SUMMARY

A kinetic study of sodium dependent GABA transport has been made in synaptosomes from 30-month old Long-Evans rats and compared to results from 2-month old animals. Initial velocity of uptake was measured as a function of both sodium and GABA concentration, and these data were then fitted to the model which was found to give best fit for the 2-month data. An excellent fit was also obtained for the 30-month data. Thus there has been no change with age in the fundamental mechanism by which carrier, sodium, and GABA interact in the process of transport. However, quantitative changes were found to occur with age both in initial velocity of uptake and in those constants which quantitate the model. The rate equation for the model was utilized along with the best fit constants to define and calculate certain parameters which were then used to quantitatively compare the transport mechanism in young and aged animals.

Acknowledgements—This work was supported by grant number NS-12292 from the National Institutes of Health and by the Medical University of South Carolina Biomedical Research Support Grant, 1981–1982. The author would like to thank Mrs. Anne L. Hungerford and Mrs. Donna W. Clark for their technical assistance.

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The prize will be awarded every second year from 1983 onwards. Individuals, research teams or institutions are invited to submit their applications in English not later than 30 September 1982 to Prof. M. Bergener, M.D., Secretary General, International Association of Gerontology, Rheinische Landesklinik, Wilhelm-Griesinger-Str. 23, D-5000 Cologne 91, Germany.

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Experimental Gerontology, Vol. 17, pp. 89–94, 1982
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LIPOFUSCIN PIGMENT ACCUMULATION IN THE CENTRAL NERVOUS SYSTEM OF THE MOUSE DURING AGING

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(*Received* 12 *March* 1981)

Abstract—Histopathological and autofluorescence investigations were carried out to study lipofuscin pigment accumulation in various age groups of mice. Qualitative studies revealed an increase of lipofuscin pigment accumulation in old animals. Quantitative studies showed a significant increase in the percentage of pigmented nerve cells and the percentage of cytoplasmic areas occupied by lipofuscin pigment granules with advancing age.

INTRODUCTION

LIPOFUSCIN PIGMENT accumulation has been observed to increase with age in human beings (Brody, 1960; Samorajski *et al.*, 1964), dogs (Whiteford and Getty, 1966; Few and Getty, 1967), pigs (Whiteford and Getty, 1966; Few and Getty, 1967; Nanda and Getty, 1971), mice (Samorajski *et al.*, 1968), rats (Reichel *et al.*, 1968; Brizzee and Johnson, 1970) and monkeys (Brizzee *et al.*, 1974). Mostly these studies revealed semi-quantitative evidence for lipofuscin increase with age except those of Reichel *et al.* (1968), Samorajski *et al.* (1968) and Brizzee *et al.* (1974) whereas Strehler *et al.* (1959) and Goyal (1981) reported quantitatively that fluorescent pigment granules accumulate linearly with time in the human ventricular myocardium.

The present study describes the results of a quantitative study of lipofuscin pigment accumulation in the central nervous system of the mouse.

MATERIALS AND METHODS

The female mice of inbred 'strain A' were procured from the mouse colony of Chittaranjan National Cancer Research Centre, Calcutta, India. They were fed the standard laboratory diet of Hindustan Lever Ltd. The studies were performed on animals of 5, 10, 15 and 20 months of age. Each was stunned to death and the cerebral cortex was immediately removed. The tissue was washed, dehydrated, cleared, and embedded in paraffin. Paraffin sections were cut at a thickness of 4 μ. The unstained sections were examined under the blue light fluorescence, after deparaffinization, in an Ortholux Carl Zeiss fluorescence microscope. Histological studies were performed on sections stained with Sudan black B and alcian blue/periodic acid Schiff.

For estimation of the percentage of neurons containing pigment granules, nerve cells were randomly selected for Sudan black B stained sections and examined under the light microscope. The percentage of neurons containing pigment granules was obtained by an examination of 100 cells from each slide.

The percentage of cytoplasmic area occupied by lipofuscin pigment granules was measured by randomly draw-

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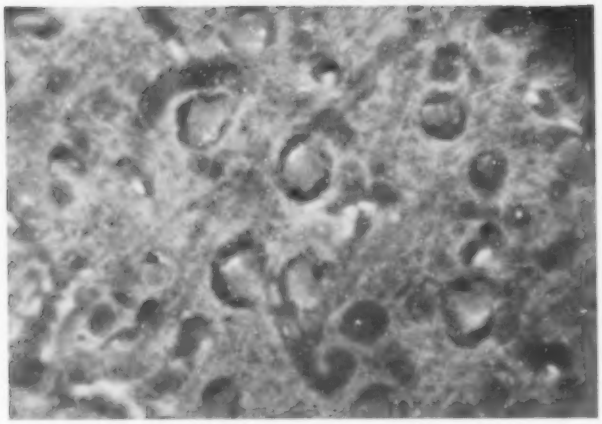

FIG. 1. Age 5 months. Fluorescence photomicrograph shows very few lipofuscin pigment granules at the nuclear periphery in nerve cells. Autofluorescence × 756.

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ing twenty camera lucida diagrams from each Sudan black B stained slide on mm² graph paper under the oil immersion field of the microscope. The percentage of area occupied by pigment granules in the cytoplasm was then calculated.

OBSERVATIONS

Histopathological and autofluorescence investigations were carried out to study qualitative as well as quantitative intracellular lipofuscin pigment accumulation with advancing age.

Qualitative observations

Lipofuscin pigment accumulation was examined in four age groups *viz.*, 5, 10, 15 and

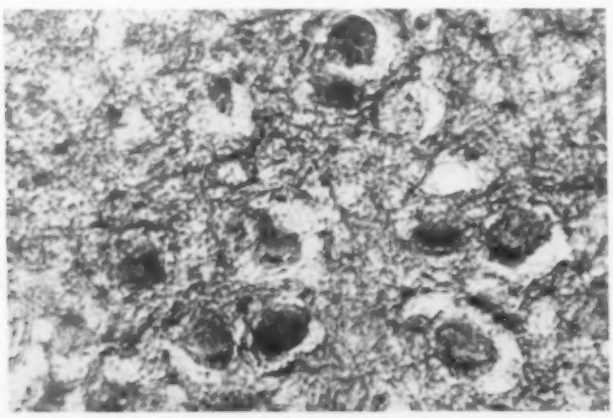

FIG. 2. Age 10 months. Photomicrograph shows lipofuscin pigment granules at the nuclear periphery in nerve cells. Sudan black β × 1200.

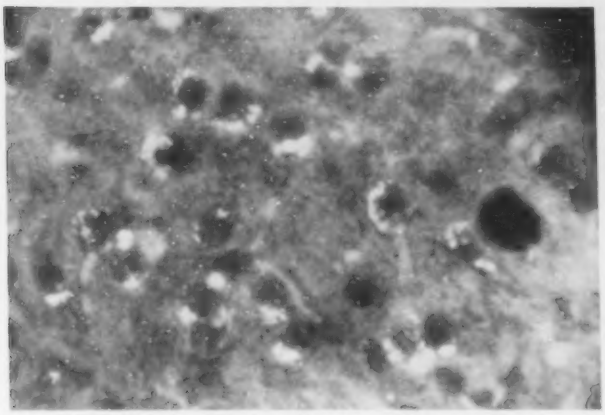

FIG. 3. Age 20 months. Fluorescence photomicrograph shows intense accumulation of lipofuscin pigment granules at the nuclear periphery in nerve cells. Autofluorescence × 756.

20 months old animals. The nerve cells at 5 months of age were found to contain very few lipofuscin pigment granules distributed in the cytoplasm near the nuclei (Fig. 1). By 10 months of age, the pigment granules were found to increase (Fig. 2). The pigment granules increased further and started concentrating at 15 months of age. At 20 months of age, the pigment granules were observed to be concentrated near the nuclear periphery and appeared to be larger in size. (Fig. 3). A progressive intracellular lipofuscin pigment accumulation was observed as a constant feature of these neurocytological changes.

On examination of nerve cells under ultraviolet light, the pigment granules exhibited light yellow fluorescence in young animals and intense bright yellow in old ones. When the fluorescence was compared with staining reactions in the same series of sections, the fluorescence was found to correspond to the stained areas of histochemical reactions.

Quantitative observations

Quantitative summaries of the percentage of pigmented nerve cells and percentage of cytoplasmic area occupied by lipofuscin, pigment granules in four age groups are shown in the Table 1 and Table 2 respectively. The quantitative increase in intracellular pigment

TABLE 1. CHANGES WITH AGE IN THE PERCENTAGE OF NERVE CELLS THAT CONTAIN LIPOFUSCIN PIGMENT GRANULES

Age Group	Age	Number of animals	Percentage of pigmented nerve cells
A	5 months	8	26.88 ± 1.83
B	10 months	8	45.13 ± 1.84
C	15 months	8	50.88 ± 2.16
D	20 months	8	69.75 ± 2.82

$P < 0.001$ = A and B, A and C, A and D, B and D, C and D. NS = B and C.

TABLE 2. CHANGES WITH AGE IN THE PERCENTAGE OF AREA OCCUPIED BY LIPOFUSCIN PIGMENT GRANULES IN THE CYTOPLASM OF NERVE CELLS

Age Group	Age	Number of animals	Percentage of area occupied by lipofuscin pigment bodies in the cytoplasm
A	5 months	8	2.27 ± 0.2651
B	10 months	8	3.92 ± 0.3725
C	15 months	8	5.47 ± 0.4566
D	20 months	8	7.75 ± 0.7966

$P < 0.001$ = A and C, A and D, B and D.
$P < 0.01$ = A and B.
$P < 0.05$ = B and C, C and D.

accumulation was estimated by the increase in the percentage of pigmented nerve cells and the percentage of cytoplasmic area occupied by lipofuscin pigment granules. The results revealed that the percentage of nerve cells that contained lipofuscin pigment granules increased progressively with age (Table 1; Fig. 4). The differences in the percentage of pigmented nerve cells between 5 and 10 months, 5 and 15 months, 5 and 20 months, 10 and 20 months and 15 and 20 months-old animals were significant (< 0.001) (Table 1).

A progressive increase in the percentage of cytoplasmic area occupied by lipofuscin pigments was also observed with advancing age (Table 2; Fig. 5). The differences in the percentage of cytoplasmic pigmented area between 5 and 15 months, 5 and 20 months and 10 and 20 months-old animals were significant (< 0.001) (Table 2).

DISCUSSION

Lipofuscin pigment accumulation has been considered the most important neurocytological change with age by a number of workers (Brody, 1960; Samorajski *et al.*, 1964, 1968, Whiteford and Getty, 1966; Few and Getty, 1967; Nanda and Getty, 1971; Brizzee *et al.*,

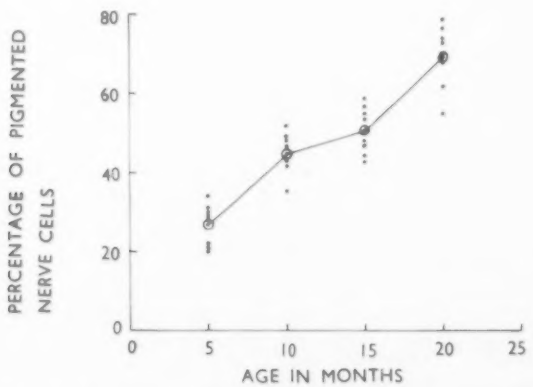

FIG. 4. Changes with age in the percentage of pigmented nerve cells.

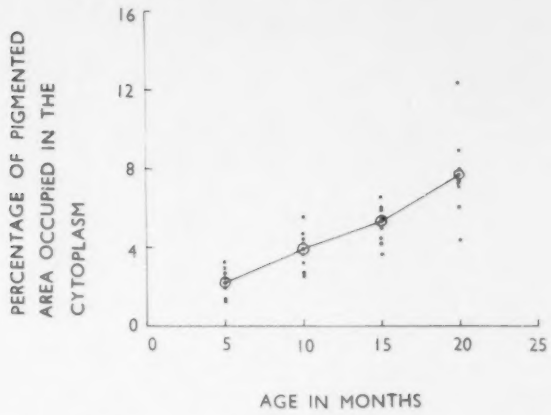

FIG. 5. Changes with age in the percentage of pigmented area occupied in the cytoplasm of nerve cells.

1974). The present study revealed a significant increase in the percentage of pigmented nerve cells and the percentage of cytoplasmic area occupied by lipofuscin pigment granules. Samorajski *et al.* (1968) observed an increase with age of cytoplasmic area occupied by lipofuscin pigment bodies in the lumbrosacral dorsal ganglia and cerebella of mice.

The distribution pattern of lipofuscin pigment granules was described as early as 1910 by Mühlmann. He observed that lipofuscin pigment granules which were distributed homogenously started accumulating in clusters and finally localized in a mass that continued to increase in size with age. The pigment was found to appear in a congregate mass in a polar pattern deep to the base of apical dendrites in pyramidal layers of hippocampus in rats and monkeys (Brizzee *et al.*, 1969; Brizzee and Johnson, 1970; Brizzee *et al.*, 1974). Whiteford and Getty (1966) described four types of distribution patterns of lipofuscins (homogenous, perinuclear, polar and bipolar) in the nerve cells of dogs. The present study showed the appearance of lipofuscin pigment in nerve cells at the perinuclear position. Thereafter lipofuscin pigment granules increased with age and started accumulating at the perinuclear position.

As the nerve cells do not divide, their loss in an aging nervous system is often associated with intraneuronal lipofuscin pigment accumulation. Samorajski *et al.* (1968), Zeman (1974), and Brizzee *et al.* (1975) suggested that accumulation of lipofuscin pigments in nerve cells may cause loss of nerve cells. A considerable loss of nerve cells has been observed in mice (Johnson and Erner, 1972) and human beings (Brody, 1970; Ball, 1977). Brody (1970) reported 50% cell loss in superior frontal gyrus of human beings with advancing age. The present investigation revealed a considerable accumulation of lipofuscin pigment in nerve cells with age. A constant and progressive accumulating of lipofuscin pigments in nerve cells indicates an aging process. It therefore seems reasonable to assume that the presence of pigment occupying most of the cytoplasmic area in nerve cells may be detrimental to normal cellular metabolism. The physiological and metabolic functions of nerve cells may probably be affected because of reduction in cytoplasmic area in old age.

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Experimental Gerontology, Vol. 17, pp. 95–103, 1982
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ACTIVITY AND MODULATION OF ORNITHINE DECARBOXYLASE AND CONCENTRATIONS OF POLYAMINES IN VARIOUS TISSUES OF RATS AS A FUNCTION OF AGE

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(*Received* 22 *March* 1981)

Abstract—The activities of ornithine decarboxylase (ODC) on the soluble and nuclear fractions of the cerebral cortex, heart and lungs of 4- (young), 38- (adult) and 85-week (old) male rats were studied. Also, the effects of aminophylline, histamine and estradiol on the activity of soluble ODC have been determined *in vitro* using slices of these tissues. The activity of ODC is significantly higher in the soluble fraction of all the tissues in comparison to that of nuclear fraction. Its activity in both the fractions is highest in the immature and decreases with increasing age in all the tissues except in the nuclear fraction of the lungs in which it increases with age. The ODC of the heart, lungs and cerebral cortex appear to be different as seen from the differences in their sensitivities to aminophylline, histamine and estradiol. In general, there is a decrease in its sensitivity to the three effectors with increasing age. This may be due to a decrease in the receptors and a concomitant decrease in ODC activity. A direct relationship between ODC activity and polyamine levels of the brain exists at various ages of the rat.

INTRODUCTION

POLYAMINES HAVE been implicated in the control of cellular growth and differentiation (Tabor and Tabor, 1976; Janne *et al.*, 1978) and RNA and protein synthesis (Kay and Pegg, 1976). They are cations and act at the chromatin level by binding with DNA phosphate (Tabor, 1962). Thereby they may alter the structure of chromatin and also its template activity. An increase in the synthesis of polyamines reflects a higher activity of ornithine decarboxylase (ODC, L-ornithine decarboxylase, 4.1.1.17), the first and the rate limiting enzyme in the polyamine biosynthetic pathway (Tabor and Tabor, 1976).

Ornithine decarboxylase is, therefore, important for cellular growth and differentiation (Tabor and Tabor, 1976; Janne *et al.*, 1978). It stimulates DNA synthesis (Moruzzi, 1975; Raina and Janne, 1970), protein synthesis (Kay and Pegg, 1976), phosphorylation of nonhistone chromosomal proteins (Imai, 1975; Faron-Furnstenthal and Lightholder, 1978), and other metabolic functions. Therefore, alterations in the activity of ODC may affect

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the levels of polyamines and thereby cause alterations in cellular metabolism during the life span of an organism.

The activity of ODC is reported to be enhanced by certain physiological agents, for example, cyclic AMP (Byus *et al.*, 1976; Insel and Fenno, 1978) and sex hormones to promote growth (Beck *et al.*, 1972; Theoharides and Canellakis, 1975). So it was considered of interest to study its activity in relation to certain effectors as a function of age. Aminophylline was selected as it increases the endogenous cAMP concentration by inhibiting tissue phosphodiesterase (Appleman *et al.*, 1973). Histamine, whose action is mediated by H_1 and H_2 receptors (Ganellin, 1978), was selected because a qualitative change in histamine action may throw some light on the relative changes in H_1 and H_2 receptor populations as a function of age. Estradiol was selected as a natural female sex hormone especially because its receptor level has been shown to decrease with age (Kanungo *et al.*, 1975).

The present study was undertaken to study (1) the activity of ODC of various tissues of the rat as a function of age, (2) the modulation of its activity by aminophylline, histamine and 17β-estradiol, and (3) to correlate its activity with the levels of polyamines.

MATERIALS AND METHODS

Animals

Immature (4-), adult (38-) and old (85-week) male rats of Wistar strain were used. They were maintained at 24 ± 2°C with artificial illumination for 12 h followed by a dark period of 12 h. They were fed on a diet containing milk powder and wheat flour (4:1, v/v) prepared daily, supplemented with gram (*Cicer arietinum*). Tap water was given *ad libitum*.

Chemicals

All the biochemicals were of analytical grade and were purchased from Sigma Chemical Co., U.S.A. except hyamine hydroxide, PPO (2,5-diphenyloxazole) and POPOP (1,4-bis(2,5-phenyloxazolyl)benzene) which were purchased from Koch-Light Laboratories Ltd., England. DL-1-^{14}C-ornithine (sp. act., 59 mCi/mmole) was purchased from Radiochemical Centre, Amersham, England. Glass distilled water was used in all experiments.

Tissue preparation for enzyme studies

The rats were killed by cervical dislocation at a fixed time (1600 h) of the day to avoid possible circadian rhythm of the enzyme. The brain, heart and lungs were taken out immediately and washed in cold normal saline to remove the adhering blood, and then blotted. The cerebral cortex of the brain and the ventricles of the heart were removed. The lungs were taken out and cleaned of cartilaginous trachea. The tissues were cut into slices of 0.2–0.4 mm thickness with the help of a clean blade (Takagaki, 1959).

In vitro study of the effects of effectors was carried out by transferring 0.1 g of the sliced tissue into Warburg flasks containing 4.0 ml of incubation medium (Krebs and Henseleit, 1932). The incubation medium was prepared by bubbling a mixture of 95% O_2 and 5% CO_2 into Krebs-Ringer bicarbonate buffer and adjusting the pH to 7.4. The medium contained 0.1 μmole/ml of pyridoxal-5'-phosphate. The flasks were then incubated in a water bath at 37°C for 15 min with shaking. Then the effectors (aminophylline, histamine or 17β-estradiol – final concentration, 4×10^{-9}M) were added to the flasks and the incubation was continued for 60 min at 37°C with shaking. Control flasks were set up without effectors.

Preparation of subcellular fractions

After the incubation period, the slices were washed twice in fresh incubation medium to remove the effectors. A 10% homogenate of the slices was made in ice-cold 0.32 M sucrose in the case of the cerebral cortex, and in 0.25 M sucrose for the cardiac muscle and lungs. The homogenate was centrifuged at 1,000 × *g* in an IEC PR-6 model refrigerated centrifuge to obtain the nuclear fraction. The supernatant was centrifuged at 105,000 × *g* for 60 minutes in a VAC 601 ultracentrifuge. The supernatant so obtained constituted the soluble fraction. The nuclear pellet was washed twice and suspended in 3 ml of the homogenizing medium. The nuclear and soluble fractions were used as the enzyme source.

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Enzyme assay

Radiometric assay of ODC of the two fractions of the cerebral cortex, cardiac muscle and lungs was carried out by a slight modification of the method of Russell and Snyder (1968). Air-tight vessels, each with a central well and a side arm, were used. The centre well contained 0.5 ml of hyamine hydroxide to trap $^{14}CO_2$ that evolved during the reaction. The side arm contained 0.5 ml of 4.0 M citric acid. The main chamber contained 2.0 ml of the reaction mixture (14.0 μmole tris-HCl buffer, pH 7.4; 0.1 μmole pyridoxal phosphate; 3–5 mg protein and 0.2 μmole L-ornithine-^{14}C). The reaction was started by adding ^{14}C-ornithine immediately after which the flasks were incubated at 37°C for 30 min. The reaction was stopped by adding citrate from the side arm. The vessels were then incubated at 37°C for a further 30 min to ensure complete absorption of $^{14}CO_2$ by hyamine hydroxide. Then hyamine hydroxide was transferred to scintillation vials containing 10 ml of toluene scintillator. The radioactivity was counted in an LS-100C Beckman liquid scintillation counter. Blanks were run in parallel using denatured enzyme. The protein content was estimated (Lowry *et al.*, 1951), and the specific activity of the enzyme was calculated as picomoles of $^{14}CO_2$ evolved/30 min/mg protein.

Estimation of polyamines in cerebral cortex

The concentrations of putrescine, spermidine and spermine of the cerebral cortex of 4-, 38- and 85-week old male rats were estimated. Extraction of polyamines from the cerebral cortex was carried out by the method of Morris *et al.* (1969). 1.0 g tissue was homogenized in 0.3 M TCA and cetrifuged at 10,000 × *g* for 30 min. The supernatant was extracted thrice with ether, neutralized and applied on a Dowex-50 (H^+ form) column (2% cross-linked, 200–400 mesh, 8.0 × 0.6 cm) for polyamine fractionation (Tabor *et al.*, 1958). Fractions of 5.0 ml were collected. The concentrations of putrescine, spermidine and spermine were determined spectrophotometrically using the 2,4-dinitrofluorobenzene method (Dubin, 1960).

RESULTS

The activity of ODC of the soluble fraction of the three tissues of the young rat is nearly 15-fold greater than that of the nuclear fraction. Except for the nuclear fraction of the lungs, the enzyme activity in both the fractions is highest in the young and decreases sharply (≈ 50%) up to adulthood, after which either no further decrease or a slow decrease occurs. In the nuclear fraction of the lungs, there is a gradual increase in its activity with increasing age (Table 1).

Aminophylline and estradiol stimulate the soluble enzyme of cerebral cortex at all ages, the maximum stimulation being in the adult. Histamine markedly inhibits the activity of

TABLE 1. SPECIFIC ACTIVITY OF SOLUBLE AND NUCLEAR ORNITHINE DECARBOXYLASE OF CEREBRAL CORTEX, HEART, AND LUNGS OF MALE RATS OF DIFFERENT AGES

Tissue	Age (weeks)	Soluble		Nuclear	
		Mean S.D.	*P*	Mean S.D.	*P*
Cerebral cortex	4	102.01 ± 16.81		6.12 ± 0.98	
	38	50.50 ± 3.58	<0.01	2.85 ± 0.26	<0.01
	85	40.65 ± 0.85	<0.01	2.62 ± 0.26	NS
Heart	4	558.22 ± 57.86		43.16 ± 4.96	
	38	243.72 ± 16.27	<0.001	10.44 ± 1.43	<0.001
	85	101.76 ± 9.48	<0.001	11.01 ± 0.71	NS
Lung	4	460.31 ± 48.99		30.31 ± 1.73	
	38	232.32 ± 10.69	<0.01	45.00 ± 6.64	<0.02
	85	220.11 ± 11.54	NS	61.01 ± 2.10	<0.02

Specific activities of ODC are given as pmoles of $^{14}CO_2$ liberated/30 min/mg protein. Each point represents the mean ± *S.D.* of triplicate determinations done on samples from 4–5 rats. *P* values less than 5% were taken as significant. *S.D.*, standard deviation; *P*, level of significance; NS, not significant; NE, no effect.

TABLE 2. EFFECTS OF AMINOPHYLLINE, HISTAMINE, AND ESTRADIOL ON SPECIFIC ACTIVITY OF ODC OF CEREBRAL CORTEX OF RATS OF DIFFERENT AGES

Experimental condition	4 weeks		38 weeks		84 weeks	
	Mean S.D.	P	Mean S.D.	P	Mean S.D.	P
Slice	102.01 ± 17.81		50.50 ± 3.58		40.65 ± 0.85	
Slice + Am.	160.10 ± 25.82 (+57%)	<0.02	99.27 ± 7.68 (+97%)	<0.001	67.51 ± 0.45 (+66%)	<0.001
Slice + His.	28.00 ± 4.08 (−73%)	<0.001	283.24 ± 6.30 (+461%)	<0.001	63.88 ± 6.22 (+57%)	<0.01
Slice + Est.	150.11 ± 25.82 (+47%)	<0.05	91.33 ± 6.90 (+81%)	<0.001	65.82 ± 4.54 (+62%)	<0.001

Specific activities of ODC are given as pmoles of $^{14}CO_2$ liberated/30 min/mg protein.

Each point represents the mean ± *S.D.* of triplicate determinations done on samples from 4–5 rats. *P* values less than 5% were taken as significant. *S.D.*, standard deviation; *P*, level of significance; NS, not significant; NE, no effect.

Concentrations of aminophylline (Am.), histamine (His.), and estradiol (Est.) were 4 × 10^{-9}M in each case. The activities were estimated in soluble fractions only.

soluble ODC of young rats, but stimulates it by nearly five-fold in the adult. Its stimulatory effect in the old is greatly reduced (Table 2).

Aminophylline stimulates ODC by more than 10-fold in the hearts of young rats and this effect sharply decreases in adults. It has a slight inhibitory effect in the old. The activity of soluble ODC of young rats is inhibited considerably by histamine. It has, however, no effect in the heart of adult and old rats. The stimulatory effect of estradiol also decreases sharply with increasing age (Table 3).

Aminophylline does not have any significant effect on its activity in the lungs of immature rats, but shows a slight stimulatory effect in adults, which increases thereafter. The

TABLE 3. EFFECTS OF AMINOPHYLLINE, HISTAMINE, AND ESTRADIOL ON SPECIFIC ACTIVITY OF ODC OF THE HEARTS OF RATS OF DIFFERENT AGES

Experimental condition	4 weeks		38 weeks		85 weeks	
	Mean S.D.	P	Mean S.D.	P	Mean S.D.	P
Slice	558.22 ± 57.86		243.72 ± 16.27		101.76 ± 9.48	
Slice + Am.	6151.97 ± 399.98 (+1002%)	<0.001	370.23 ± 20.27 (+52%)	<0.001	83.09 ± 5.96 (−18%)	<0.05
Slice + His.	177.83 ± 26.00 (−68%)	<0.001	223.43 ± 21.37 (NE)	NS	100.56 ± 8.26 (NE)	NS
Slice + Est.	1269.02 ± 112.79 (+127%)	<0.001	357.11 ± 19.99 (+47%)	<0.001	126.99 ± 12.55 (+25%)	<0.05

Specific activities of ODC are given as pmoles of $^{14}CO_2$ liberated/30 min/mg protein. Each point represents the mean ± *S.D.* of triplicate determinations done on samples from 4–5 rats. *P* values less than 5% were taken as significant. *S.D.*, standard deviation; *P*, level of significance; NS, not significant; NE, no effect.

Concentrations of aminophylline (Am.), histamine (His.), and estradiol (Est.) were 4 × 10^{-9}M in each case. The activities were estimated in soluble fractions only.

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TABLE 4. EFFECTS OF AMINOPHYLLINE, HISTAMINE, AND ESTRADIOL ON SPECIFIC ACTIVITY OF ODC OF THE LUNGS OF RATS OF DIFFERENT AGES

Experimental condition	4 weeks		38 weeks		85 weeks	
	Mean S.D.	P	Mean S.D.	P	Mean S.D.	P
Slice	460.31 ± 48.99		232.32 ± 10.69		220.10 ± 11.54	
Slice + Am.	460.04 ± 40.20 (NE)	NS	260.06 ± 20.39 (+12%)	NS	273.15 ± 29.81 (+24%)	<0.05
Slice + His.	1930.03 ± 181.03 (+319%)	<0.001	380.07 ± 26.88 (+64%)	<0.001	298.25 ± 17.92 (+36%)	<0.001
Slice + Est.	1090.14 ± 113.74 (+137%)	<0.001	310.32 ± 23.18 (+34%)	<0.01	278.25 ± 25.37 (+26%)	<0.02

Specific activities of ODC are given as pmoles of $^{14}CO_2$ liberated/30 min/mg protein. Each point represents the mean ± *S.D.* of triplicate determinations done on samples from 4–5 rats. *P* values less than 5% were taken as significant. *S.D.*, standard deviation; *P*, level of significance; NS, not significant; NE, no effect.

Concentrations of aminophylline (Am.), histamine (His.), and estradiol (Est.) were 4×10^{-9}M in each case. The activities were estimated in soluble fractions only.

stimulatory effect of histamine on ODC is maximum (4-fold) in young rats and sharply decreases with age. Estradiol stimulates ODC by nearly two-fold in the young. This effect decreases markedly with increasing age (Table 4).

The elution pattern of brain polyamines (Fig. 1) shows that ornithine gets eluted at the bed volume. Putrescine and other polyamines get eluted with increasing concentration of HC1. Putrescine is eluted between 0.25 N–0.5 N HC1, spermidine between 1.0–1.25 N HC1 and spermine between 1.5–1.75 N HC1.

No significant difference with age in the putrescine content is observed (Fig. 2). However, the spermidine and spermine concentrations decrease sharply with increasing age. The spermidine content in the old is 33% of that of the adult and only 12% of that of the young. The spermine content of the old is 50% of that of the adult and 33% of that of the young.

DISCUSSION

The activity of ODC is 5-to-20-fold higher in the soluble fraction than in the nuclear fraction of the three tissues at the three ages studied (Table 1), which was also observed earlier (Murphy and Brosnau, 1976). This clearly shows that ODC is predominantly a cytosolic enzyme which is in agreement with the finding of Janne and Raina (1968). ODC catalyzes the first and the rate limiting step in the biosynthetic path of polyamines. So it appears that polyamines are synthesized in the cytoplasm and then translocated into the nucleus where they may interact with chromatin, thereby regulating cellular metabolism (Janne & Raina, 1968; Kremzner, 1970). So any change in the activity of soluble ODC may affect polyamine level and, therefore, cellular metabolism.

The activity of ODC in the heart and lungs is much higher than in the cerebral cortex at all ages. The activity of ODC in the soluble fraction of the three tissues decreases sharply between immature and adult age. The higher activity of ODC in immature rats corresponds to the phase of rapid cellular growth. The reason for a gradual increase with age in the activity of nuclear ODC of the lungs is not clear.

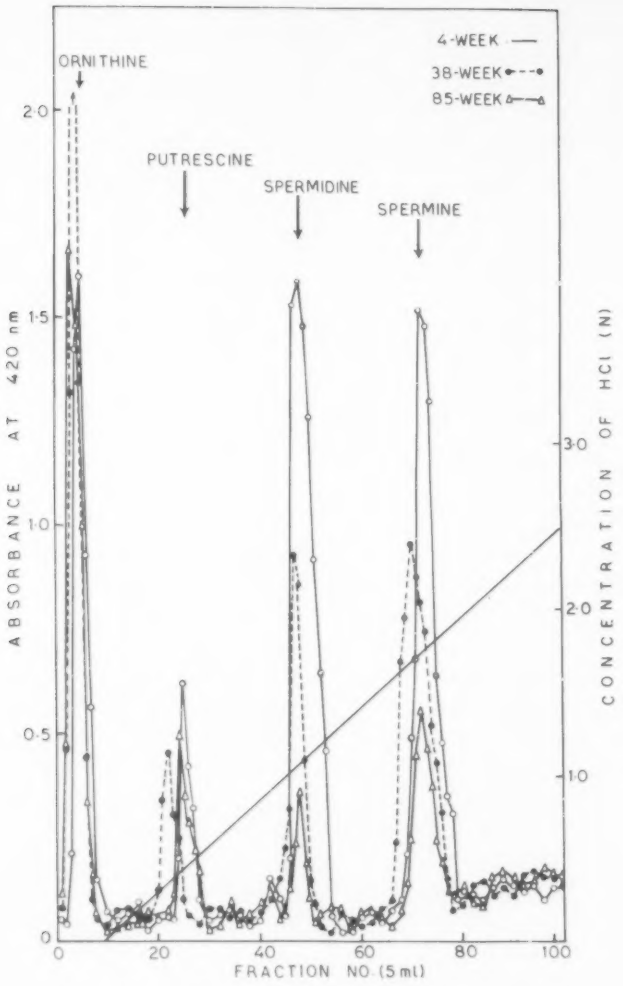

FIG. 1. The elution pattern of brain polyamines.

Aminophylline causes a rapid increase in the cAMP level by inhibiting intracellular phosphodiesterase (Appleman *et al.*, 1973). Cyclic AMP is reported to stimulate ODC activity (Aisbitt and Barry, 1973; Holtta and Raina, 1973). Therefore, the stimulation of ODC activity by aminophylline in different tissues may be mediated through cAMP. In comparison to the cerebral cortex and lungs, adrenergic activity is high in the heart and is mediated through the adenyl cyclase-cAMP system. Aminophylline-induced stimulation of ODC activity (10-fold) is also highest in the hearts of immature rats and its effect decreases rapidly with increasing age. In the cerebral cortex, aminophylline produces a mild stimulation of ODC which has no significant age-dependent variations. The effect of aminophylline on the lungs is little and does not show any significant difference with age. The data show the ODC of the heart to be most sensitive to cAMP and that of the lungs to

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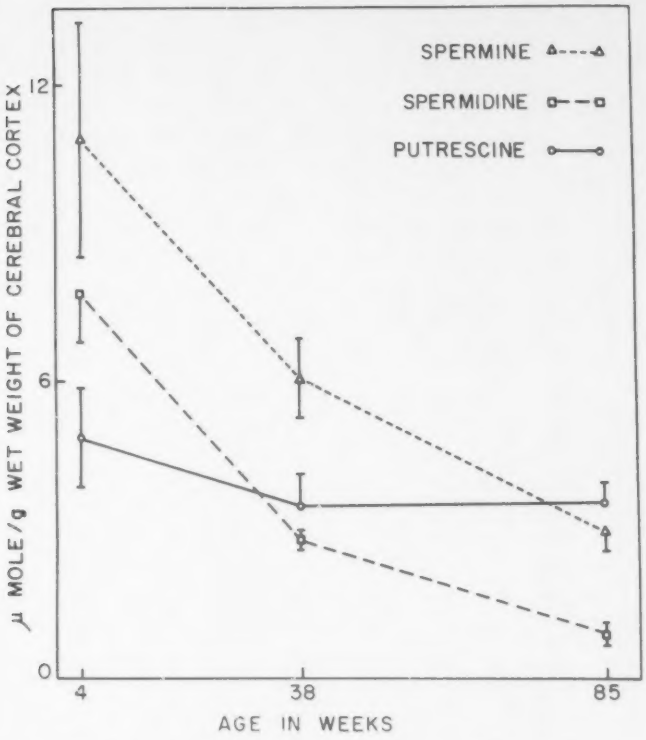

FIG. 2. The effect of aging on concentrations of brain polyamines.

be the least. In addition, the sensitivity of cardiac ODC to cAMP declines rapidly with advancing age. It is possible that the isoenzymes of ODC of the lungs and cerebral cortex are different from those of the heart.

The effects of histamine on ODC vary greatly with tissue and age. Histamine is reported to increase adenyl cyclase activity and cAMP concentration in several tissues (Kakiuchi and Rall, 1968; Klein and Levey, 1971). However, if the action of histamine is mediated through cAMP, then histamine should have only stimulatory action on ODC. Histamine activity is known to be mediated through either H_1 or H_2 receptors, the actions of which are different, if not opposite (Ganellin, 1978). Our data show that in the lungs, histamine-stimulated ODC activity is highest in the young and is the least in the old. In the heart, histamine markedly depresses ODC activity in the young. It is known that histamine receptors of the lungs are predominantly of H_1 type (Eyre, 1971) and that of the heart (ventricle) are of H_2 type (McNeill and Verma, 1974). It is possible that H_1 receptor-mediated action, as in the lungs, stimulates, and H_2 receptor-mediated action, as in the heart, depresses ODC activity. In both these tissues, however, there is a rapid decline in histamine sensitivity with increasing age. In case of the brain, histamine markedly depresses ODC activity in the young but markedly stimulates it in the adult. It is possible that the opposite actions of histamine on ODC of the brain which is age-dependent, is due to a quantitative change in the relative populations of H_1 and H_2 receptors (Lomax *et al.*, 1975). In the

young, H_2 receptor population may be high, whereas in the adult there seems to be a predominance of H_1 receptors. In old rats, there appears to be a decrease in both types of receptors.

Sex hormones are known to promote growth during development. The stimulatory action of estradiol on ODC of different tissues supports this physiological role of the female sex hormone. In the heart and lungs, estradiol-induced stimulation of ODC is highest in the young and decreases rapidly with increasing age. This age-dependent change may be related to the decrease in the estradiol receptor concentration with increasing age (Kanungo *et al.*, 1975). In the brain, however, estradiol produces a slight stimulation of ODC activity without any significant age-dependent variation. This may be because the brain isoenzymes of ODC are different from those of the heart and lungs. Different isoenzymes of ODC are reported to be present in the brains and livers of rats (Butler and Schanberg, 1976).

Concomitant with the decrease in ODC activity it is seen that the levels of polyamines, spermine and spermidine, decrease with age (Fig. 1). Higher levels of polyamines may be necessary in immature rats for rapid growth and proliferation of cells that occur at this age (Levine *et al.*, 1975; Pegg, 1977). However, no significant change in the putrescine level is seen with increasing age. It is reported that spermine gets converted to spermidine and then to putrescine (Holtta, 1977). Hence putrescine may not play any major role in differentiation, growth, and aging but may act as a pool so that whenever the levels of spermine and spermidine go down, it gets converted to these polyamines and thus maintains the metabolic functions of the cell. A decrease in the levels of spermidine and spermine with increasing age may affect RNA synthesis (Tabor and Tabor, 1976; Moruzzi *et al.*, 1975), protein synthesis (Kay and Pegg, 1973), and other metabolic functions.

Our results show that (a) the activities of ODC vary widely with the type of tissue and may be related to their metabolic and functional activities, (b) there is a sharp decrease in its activity in all the tissues during the growth period, (c) the soluble and nuclear ODC of the heart, lungs, and cerebral cortex appear to be different as indicated by the differences in their sensitivities to aminophylline, estradiol, and histamine, (d) in general, there is a decrease in the sensitivity of ODC to aminophylline, estradiol, and histamine with increasing age, (e) there appears to be a change in the proportions of histamine receptors in the cerebral cortex during aging, and (f) the concentrations of spermidine and spermine in rat brains decrease with increasing age.

It has been proposed that sequential activation and repression of genes by various modulators appearing and/or disappearing during the life span of an organism (Kanungo, 1975, 1980) may lead to aging. Our study shows that both the activity of ODC and the levels of polyamines in the brains of rats decrease with increasing age. Age-dependent variations in the levels of polyamines may alter the conformation of chromatin, and hence the expression of genes during development, growth, and senescence of an organism (Kanungo, 1975, 1978).

Acknowledgements—We thank Professor J. Janne, University of Helsinki, Finland, for the personal communication. This investigation was supported by grants to MSK from the Nuffield Foundation, England, PL-480 (INARS-24), University Grants Commission, and Department of Science & Technology, India. Ratna Das thanks the C.S.I.R. for a research fellowship.

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Experimental Gerontology, Vol. 17, pp. 105–114, 1982
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ACCELERATED AGING OF FASTED *DROSOPHILA*

Preservation of Physiological Function and Cellular Fine Structure by Thiazolidine Carboxylic Acid (TCA)

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(*Received 10 April* 1981)

INTRODUCTION

Among the various pharmacological agents that have been used with experimental animals in attempts to protect the cells and the organism from the deleterious effects of aging, work from this laboratory (Miquel and Economos, 1979) has shown that salts of thiazolidine carboxylic acid (TCA) are particularly effective in mice and *Drosophila*. In the present study we investigated in more detail the effects of TCA in one organism, *Drosophila*. Unlike other studies where the level of toxicity of the tested agent is not investigated, we determined both the minimal effective dose for a life-prolongation effect and the dose at which TCA becomes toxic. (Such information is needed to evaluate the "safety" of a compound.) These doses were studied in both *ad lib.* fed flies and in flies which, after being fed treated food for a week, were deprived of food and water and exhibited accelerated mortality kinetics and, apparently, accelerated aging. Possible preservation by TCA of cellular fine structure of fasted flies was investigated by electron microscopic study of wing muscle. Various other effects of TCA on physiological functions of the aging flies were studied. Such study of the effects of TCA at different levels of biological organization is necessary in order to understand the possible mechanisms underlying the effects of this chemical on the prolongation of life of experimental animals. At the same time this chemical becomes an investigative probe in the study of the mechanisms of aging.

Note: In a subsequent study (Miquel, Fleming and Economos, *Arch. Geront. Geriatr.* **1** (2), 1982, in press) TCA's effect on the metabolic rate of *Drosophila* was confirmed and extended to a number of other antioxidants which also prolonged *Drosophila* life span; moreover the life prolongation effect was found to correlate with the chemicals' effect on oxygen consumption rate of the flies, in agreement with the rate of living theory of aging.

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MATERIALS AND METHODS

The experimental animals were male *Drosophila melanogaster* of the Oregon R strain, developed in an incubator at 21°C in standard commercially prepared cornmeal-molasses medium, in half-pint bottles. All animals were kept on control food in the incubator until they were 3 days old, to avoid interference with the growth process during this period. The female flies used in the mating studies were virgins, collected from the stock bottles and kept on control food at 21°C until 7–9 days old.

TCA (L-thiazolidine-4-carboxylic acid) powder was added to melted nutrient medium in appropriate amounts to obtain the various desired concentrations. The food was allowed to cool for at least one day before use. A piece of dry cellucotton paper was affixed on the food to provide a dry landing place for the flies. In the fasting experiments, the flies were fed the treated food for one week at 21°C and then were placed in empty half-pint bottles and those dying were recorded hourly; the temperature during starvation depended on the experiment. In *ad lib.* fed populations, the flies were twice weekly transferred to fresh food bottles by shaking. Dead flies were recorded at each transfer.

In one mating experiment, the percent of control and experimental animals (treated with various doses of TCA for 4 weeks at 27°C) which were able to mate, was determined by shaking them into empty bottles with about twice as many virgin females. Thirteen minutes later 0.5 ml methophane was injected in the cotton placed on the internal side of the bottle-cap. When the flies were deeply anesthetized (after about 2-min of exposure to methophane), the uncoupled females were discarded, while the couples were counted and, together with the uncoupled males, were placed in different empty bottles and were left for one hour to recover from the anesthesia. By then all males had uncoupled and, under light methophane anesthesia, were transferred back to their food bottles. In the experiment in which the speed of mating was determined, five males were placed at time zero in each empty standard glass vial with ten virgin females. The males in this experiment were 3 weeks old and had been on TCA for 2½ weeks. The vials were placed horizontally on the table and were left undisturbed; the number of coupled males was recorded at 7, 15 and 20 min. At the end of the experiment the males were separated from the females using the procedure described above.

Oxygen consumption of groups of unanesthetized male flies was determined using a Gilson respirometer. During the initial 60 min, oxygen consumption was elevated and irregular due to the agitation of flies from transfer into the flasks of the respirometer. Thereafter the flies became quiet and the oxygen consumption rate became stable over at least 100 min, during which recordings were made every 20 min.

For the development studies, control males and females about 2 weeks old were left overnight at 21°C in inverted bottles with food medium on the cap. The food was divided into four quarters, and each quadrant had about 50–90 eggs. The cap was cut along the dividing lines and two quadrants were placed in each bottle with control or treated food, the side with the medium and the eggs touching the food. The bottles were placed at 27°C where the flies eclosed about 9 to 11 days later. The flies were collected daily and were kept in bottles with fresh control medium until weighed.

For the study of fine structure three groups of flies were used, one group of *ad lib.* fed control flies, and two groups of flies fasted for 25 hours after being fed for a week with control food or food containing 0.3% TCA. The time of sacrifice, 25 h, was chosen such that 60% of the control fasted flies had died with only 6% dead in the group that had been fed TCA-treated food. The procedures for obtaining and fixing the wing muscle tissues were as previously described (Miquel *et al*, 1972).

RESULTS

Fasted Drosophila

Ad libitum fed male Drosophila have a maximum life span of about 120 d at 21°C. However, when one-week old *ad lib.* fed flies are deprived of food and water, they survive less than about 36h at 21°C; their mortality kinetics have a form similar to that of the *ad lib.* fed populations, but are considerably accelerated (see Figs. 1 and 2). In these figures mortality kinetics are plotted using a recently introduced model (Economos, 1979, 1980), which represents mortality kinetics on a semilogarithmic plot by a pair of straight lines, one for increasing cumulative mortality in the first part of life span (line of non-survivors) and one for decreasing survivorship in the latter part of life span (line of survivors). The figures show that at a higher temperature mortality is accelerated in a similar way in both starved and *ad lib.* fed populations. Interestingly, if the terminal 20% survivors of a population of young male flies starved at 25°C are returned to food bottles, they ex-

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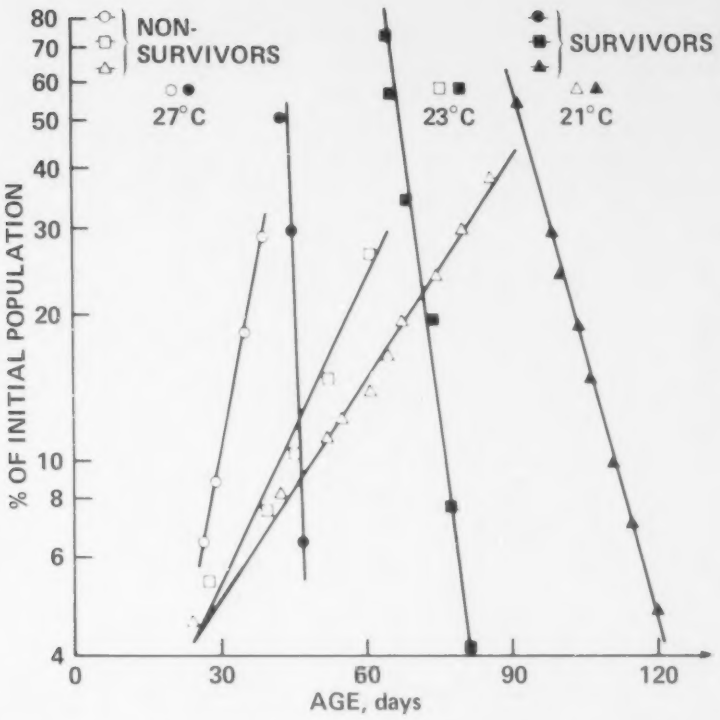

FIG. 1. Mortality kinetics of *ad lib.* fed male *Drosophila* at three different temperatures, plotted using a recently introduced model (Economos, 1979, 1980). The line of non-survivors represents cumulative mortality, the line of survivors represents survivorship. The temperature is seen to affect the slope of the various lines (rate of mortality).

perience accelerated mortality and reduced life span compared with controls not exposed to fasting (Fig. 3), indicating that fasting causes an irreversible cellular damage.

TCA dose-response study

The concentration level in the food of *Drosophila* that is "safe" (*i.e.*, non-toxic) and maximally effective (*i.e.*, it has maximal life-prolongation effect) was determined by administering the chemical at various doses, from 0.06% to 3.0% to groups of 100 male flies at 25°C. (see Fig. 4). It appears that the dose 0.06% was hardly effective, 0.3% was optimal (a large prolongation of mean and maximum life span), while above 0.6% TCA was toxic. When TCA was administered for 1 week in the food of groups of male flies which were then deprived of food and water, similar results were obtained (Fig. 5). However, 0.45% had a slightly larger effect than 0.3% in prolonging life span, while 0.6% had apparently a favorable rather than a mild toxic effect on survivorship. That TCA appeared to be toxic already at 0.6% in *ad lib.* fed flies may be due to the high concentration of the mold inhibitor benzyl benzoate that had been inadvertently added to the commercially prepared food medium.

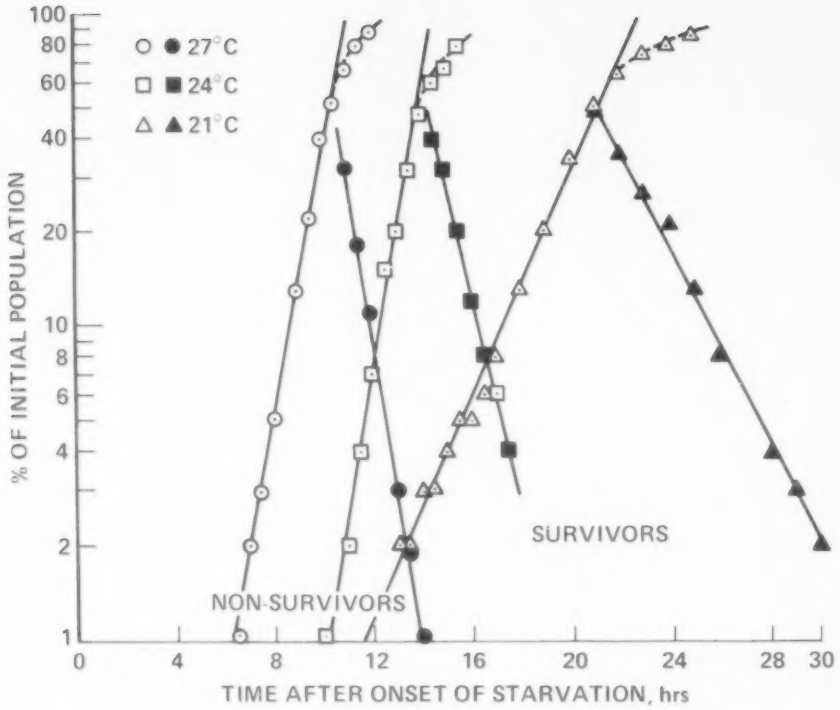

FIG. 2. Mortality kinetics of male *Drosophila* fed control food for 1 week at 21°C and then deprived of food and water at three different temperatures. The form of mortality kinetics of starved flies is similar to that shown in Fig. 1 for *ad lib.* fed flies, except that besides change of slope of the lines with temperature, there is a small shift of the non-survivors line toward shorter survival at the highest temperature.

Physiological effects of TCA

In order to study the *mechanism* of the TCA-effects on life span, we measured, at 27°C, the oxygen consumption rate (using a respirometer) of young control male flies and flies receiving 0.3% TCA in the food for about 2 weeks (Table 1). TCA-treatment reduced the specific metabolic rate (oxygen consumption rate) of the flies by about 20% without any apparent effect on their general physiology and activity. This was ascertained by measuring the mating capacity of treated and control flies at young adult age. We found that 0.3% TCA preserved the mating capacity of the male flies (Table 2), while this capacity was clearly reduced in a population treated with 0.9% TCA which is in the range of toxic doses (Fig. 4). Furthermore, we found that the speed of mating was much better preserved in the 0.3% TCA-treated flies than in controls (Table 3). Though at steady-state (15–25 min) the control and experimental groups achieved approximately the same mating score, the flies on TCA were faster in achieving mating (77.5% *vs.* 45% controls at 7 min), which is characteristic of younger age.

Eggs from control populations of flies appeared to develop somewhat slower in TCA-

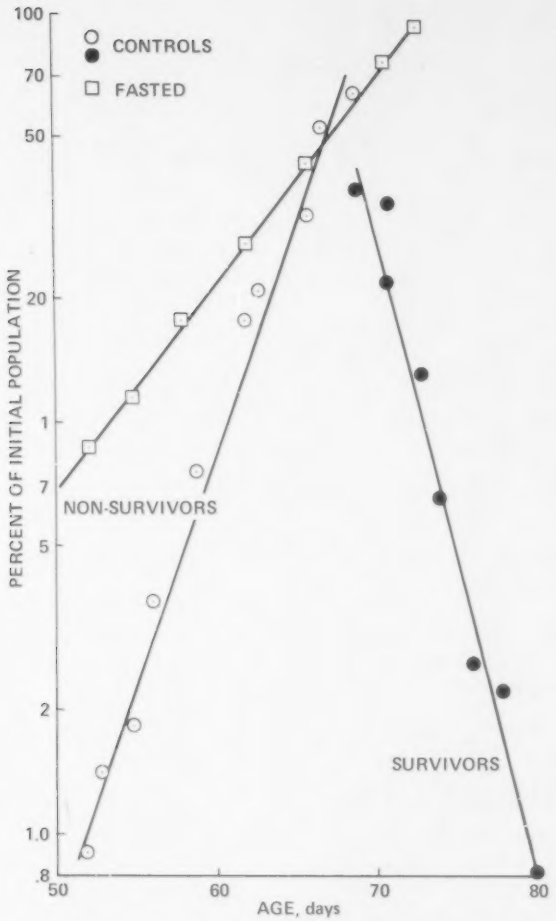

FIG. 3. Effect of starvation up to the level of 20% survivorship on subsequent mortality kinetics of male *Drosophila* with food *ad lib.* as compared with controls that were not exposed to starvation.

treated food, while the size of the eclosed flies was considerably reduced (Table 4); the effect was dose-dependent.

Fine-structural effects of starvation and TCA

Fig. 6 illustrates the appearance of wing muscle fine structure in control, *ad lib.* fed young male flies compared with flies that were first on control food or on food with 0.3% TCA and then were starved for 25 hours prior to sacrifice. Apparently, retardation of mortality by previous feeding with TCA is paralleled by relative preservation of fine structure.

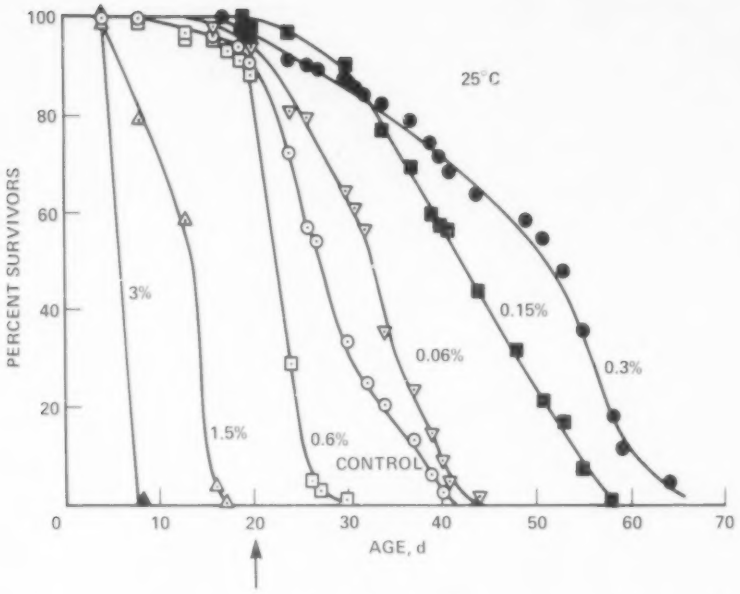

FIG. 4. Survivorship curves at 25°C for *ad lib.* fed male flies, which were fed medium containing TCA at various concentrations. (As discovered at the end of the experiment, from age 20 d on [arrow] the commercially prepared food inadvertently had a high concentration of the mold inhibitor benzyl benzoate.)

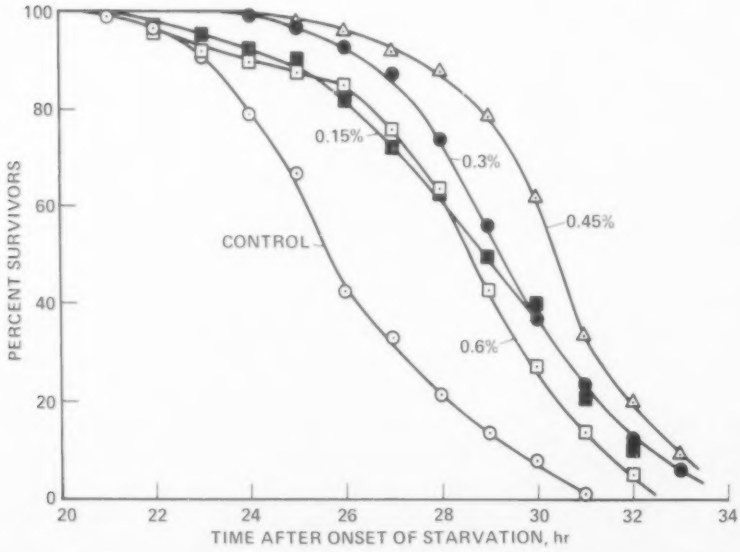

FIG. 5. Survivorship of male flies deprived of food and water at zero time at 25°C. The flies were previously fed for 1 week at 21°C medium with TCA at various concentrations and were about 10 d old at the time of starvation.

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TABLE 1. EFFECT OF TCA ON OXYGEN CONSUMPTION RATE OF AD LIB FED FLIES

Group	Number of flies	Average weight (mg)	$\dot{O}_2$ (μl/mg/day)	
				Mean ± *SD*
Control 1	33	0.743	**104	
2*	20	0.770	115	107.2 ± 7.6
3	32	0.770	101.6	
.3% TCA 1	47	0.762	85.7	
2	43	0.767	89.5	90.0 ± 3.7
3*	42	0.746	94.8	

*The flies in these groups were agitated at the beginning of the experiment due to a temporary technical problem.

**This column gives values calculated from average oxygen consumption over a period of 100 min, after the initial 60 min from placement of the flies in the respirometer. (The difference between TCA-treated and control animals is significant at the $p = 0.05$ level, student's *t*-test.)

DISCUSSION

Thiazolidine-4-carbozylic acid (TCA), a natural liver metabolite, was first synthesized chemically in 1936 by Schubert; its biological effects have been extensively investigated (Weber, unpublished review). TCA's main effect appears to be in liver protection and detoxification processes, but an anti-cancer effect has also been recently reported (Brugarolas and Gosalvez, 1980). Oeriu and collaborators have studied possible age-retardant effects of TCA in combination with folic acid in both experimental animals and aged human volunteers. Among the effects they have reported are increased life span of mice and guinea pigs (Oeriu and Vochitu, 1965) and various hormonal effects (*e.g.* Moldoveanu *et al*, 1975; for a review see Oeriu and Oeriu, 1968). As reviewed previously (Miquel and Economos, 1979), TCA is believed to act as an antioxidant, in particular it may prevent the decrease of the -SH/-SS- ratio that is seen in the senescing organisms.

In our investigations with dietary TCA, a consistent 15–20% prolongation of mean and maximal life span of *Drosophila* has been seen at various temperatures. The data reported here on the effect of TCA on resting specific metabolic rate of flies indicate that the agent may have also other than antioxidant effects. The 20% reduction in oxygen consumption rate does not seem to compromise the physiological capacity of the flies as seen from the unimpaired mating capacity of the flies; on the contrary, the flies achieve mating faster than controls, thus resembling younger flies. A possible explanation for these effects of TCA might be that it prevents "waste" of metabolic energy in heat, for instance by improving mitochondrial coupling. Increased mitochondrial efficiency may be responsible

TABLE 2. EFFECT OF VARIOUS DOSES OF TCA ON MATING CAPACITY OF MALE FLIES

Group	Number of males	Number of females	Number of couples at 13 min	% of males mated
Control	32	60	14	43.7
TCA 0.3 %	86	150	34	39.5
0.45%	45	75	26	57.8
0.6 %	42	75	19	45.2
0.9 %	30	50	6	20.0

TABLE 3. EFFECTS OF 0.3% TCA ON MATING SPEED OF MALE FLIES

Vial number	Time (min)			Vial number	Time (min)		
	7	15	20		7	15	20
Control 1	2*	3	3	.3% TCA 1	3	4	4
2	3	4	4	2	4	4	4
3	1	1	3	3	3	3	3
4	2	4	5	4	4	3	3
5	2	4	4	5	4	5	4
6	3	4	4	6	5	5	5
7	3	5	5	7	3	3	3
8	2	5	5	8	5	5	5
Total →	18	30	33	Total →	31	32	31
% →	45.0	75.0	82.5	% →	77.5	80.0	77.5

*Number of males coupled with females at various times after males and females were placed in the same vial.

for some protection of cellular fine structure during starvation. While starved controls show complete depletion of glycogen and shrinking and densification of mitochondria, TCA appears to temporarily counteract these effects of food deprivation or at least reduce the rate of their expression.

Use of fasting as a method of accelerated aging was prompted by the observation of similar mortality kinetics of flies at various temperatures compared with *ad lib.* fed controls but on a much reduced time scale. Pearl used starvation as a method of studying "inherent vitality" of organisms (Pearl, 1928) and Ducoff *et al.* (1970) observed that aging beetles were increasingly more susceptible to starvation compared with younger individuals.

Increased sensitivity to starvation with age has also been seen in *Drosophila* (unpublished). The present study with TCA shows that the chemical affects mortality kinetics similarly in *ad lib.* fed flies and those deprived of food and water but previously fed TCA for 1 week.

The observed effects of TCA on developmental speed and size of the flies indicate a possible effect on cell division and may be related to the recently reported anti-tumor effects of the chemical (Brugarolas and Gosalvez, 1980). It appears that TCA is able to induce "reverse transformation" of tumor cells into normal cells and restore contact inhibition as well as inhibit further growth of malignant cells.

The question of possible toxicity of the various experimental antioxidants has rarely

TABLE 4. EFFECT OF TCA ON DEVELOPMENT OF FLIES

Treatment	*N* of eggs	*N* eclosed	Percent eclosed	Mean duration of development (d)	Mean weight, ♂, mg	Mean weight, ♀, mg
Control	620	250	40.3	9.6	0.78	1.05
0.3% TCA	691	301	43.6	10.0	0.68	0.89
0.6% TCA	230	115	50.0	10.8	0.63	0.79

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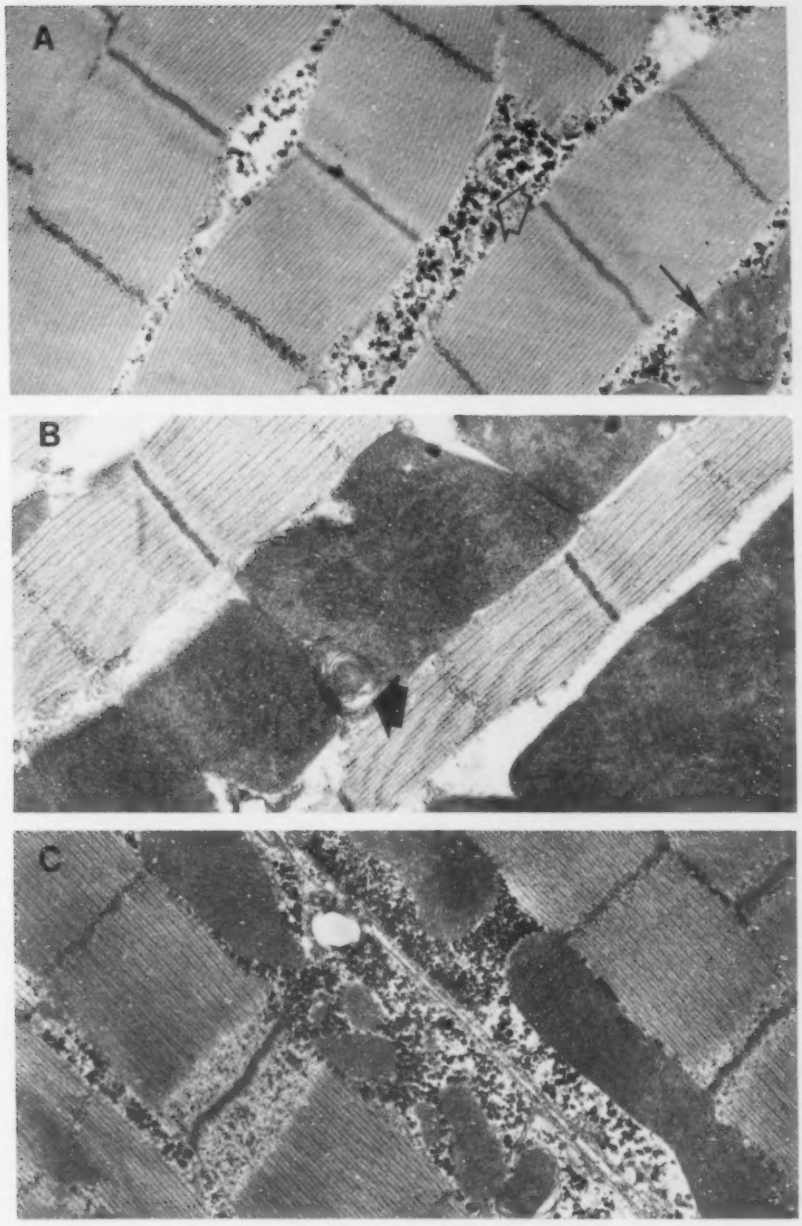

FIG. 6. Flight muscle of 10-day old male *Drosophila melanogaster* X20,550. (A) Control, *ad lib.* fed *Drosophila* showing abundant glycogen (hollow arrow) and normal mitochondria (thin arrow). (B) Flight muscle of a 10-day old fly that was maintained on normal nutrient medium and then deprived of food and water for 25 hours. Notice the starvation-induced loss of glycogen and the presence of a membranous whorl (arrow) similar to those found in non-fasted senescent flies. (C) Flight muscle of a fasted fly that had been administered 0.3% TCA in the medium for 7 days prior to starvation starting at age of 3 days. Notice the presence of abundant glycogen granules.

been addressed. Yet it is important to know how close the effective dose is to a possible threshold dose for toxicity. This "safety margin" is surprisingly small for TCA. While a concentration of 0.3% in the food is about optimal for long-term treatment and life-prolonging effect on the flies, at about three times that level mitochondrial function and other cellular functions may be depressed enough to lead to toxicity during long-term treatment. When injections of TCA are given or TCA is ingested in the form of tablets as used against liver disease, caution is needed because of possible CNS effects of large single doses (Garnier *et al.* 1980) as compared with administration spread over the entire day in ingestion with the meals. In mice, TCA at 0.2% in dry food pellets (or about 300 mg/kg/day) had no toxic effect (Miquel, unpublished), while the magnesium salt of TCA increased the mean life span of mice at a dose of about 100 mg/kg/day (Miquel and Economos, 1979). Taking into consideration the difference in specific metabolic rates between mice and humans, the life-prolonging dose for mice is equivalent to about 10 mg/kg/day for humans. For comparison, a dose of 20 mg/kg/day had clear anti-tumor effects (Brugarolas and Gosalvez, 1980).

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SUMMARY

Thiazolidine carboxylic acid (TCA) is a natural liver metabolite whose M_g-salt increased lifespan of flies and mice (Miquel and Economos, 1979, *Exp. Geront.* **14**: 279). We studied the physiological and cellular fine structural effects of various concentrations of TCA in the food of male *Drosophila*. Flies on 0.3% TCA at 27°C had a reduced oxygen consumption rate (about 20% less than controls) at 3 wks of age while their mating capacity and speed of mating were preserved; the flies lived in various experiments 20–30% longer than controls. Apparently TCA improved the metabolic efficiency of the flies (possibly from less "waste" of energy due to improved mitochondrial coupling). However, flies on 0.9% TCA had a reduced mating capacity and lifespan (:toxicity) while at 0.1% TCA was ineffective. A similar dose-response relationship was found in young flies treated with TCA for 1 week and then deprived of food and water, a procedure found to induce accelerated physiological aging. TCA at the 0.3% and 0.6% level reduced the speed of development and the size of the eclosed flies. Electron microscopic investigation of wing muscle showed that 0.3% TCA had a protective effect on cellular fine structure. Though in starved controls (40% survivors after 25 hours of starvation) there was a total absence of glycogen granules, and a striking shrinkage and densification of mitochondria, TCA to a large extent protected muscle cells from these effects of starvation.

Acknowledgements—The work of ACE in this project was supported by a grant from The Glenn Foundation for Medical Research.

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Experimental Gerontology, Vol. 17, pp. 115–120, 1982
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THE INFLUENCE OF AGING ON INTESTINAL ABSORPTION OF VITAMINS A AND D BY THE RAT

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(*Received* 27 *June* 1981)

INTRODUCTION

KNOWLEDGE OF changes in vitamin absorption that occur with age is essential in determining vitamin needs of the elderly. Several studies with human subjects have reported lower serum levels of certain vitamins in old as compared to young adults (as reviewed by Barrows and Kokkonen, 1977; Fleming, 1981). Although these findings may result from a reduced dietary intake, they may also be the result of age-associated impairments in vitamin absorption. A number of changes in the small intestine occur with age which may result in altered functional capacity of the aging gut. These include progressive replacement of normal tissue by coarse fibrous tissue (Suntzeff and Angeletti, 1961), increased generation time (Lesher *et al.*, 1961; Thrasher and Greulich, 1965) among decreased numbers of proliferative cells (Thrasher, 1971), decreased intestinal enzyme activity (Sayeed, 1967), and alterations in intestinal microflora (Gorbach *et al.*, 1967).

Studies of vitamin D supplementation suggest that increased oral doses of vitamin D may be required to maintain plasma vitamin D levels in old as compared to young adults (Lester *et al.*, 1977; MacLennan and Hamilton, 1977; Barragry *et al.*, 1978). Malabsorption of vitamin D in elderly subjects has also been suggested as a contributing factor in development of osteomalacia and osteoporosis (Barragry *et al.*, 1978; Anwar, 1978). Although there do not appear to be significant age trends in fasting serum vitamin A or carotene levels (Gillum 1955; HANES 1974; Ten State Nutrition Survey, 1972; Fisher, 1978), there are only very limited data on the influence of age on vitamin A absorption (Yiengst and Shock, 1949; Hollander and Morgan, 1979). Therefore, the present study was designed to examine the effect of age on absorption of the fat soluble vitamins A and D.

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MATERIALS AND METHODS

Animals and diets

Female Wistar rats, aged 6, 12, and 24 months, were born and reared in the Gerontology Research Center (GRC) animal colony. The rats were housed five to a 41 cm × 29 cm × 18 cm cage, maintained at a constant temperature of 72°F and exposed to a 12 hr light/dark cycle. Rats were placed in the experimental animal rooms for 2 weeks prior to the experiment under housing conditions similar to those in the GRC colony except for placement in individual cages. This 2 week period was utilized for observation of body weights and general appearance to help insure that only healthy rats were used for the experiment. In addition, this gave the rats time to adapt to laboratory conditions prior to the actual experiment. Rats were allowed free access to Purina Rat Chow® throughout their life span.

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Isotopes

Vitamin A (all Trans) [1-^{3}H (N) 1] (New England Nuclear, Boston, MA) with a specific activity of 15.6 mCi/mg and [4-^{14}C] vitamin D_3 (Amersham, Arlington Heights, IL) with a specific activity of 77.8 uCi mg were used in the experiments. Purity of the isotopes was determined by thin layer chromatography using developing systems of cyclohexane: ether (1:1) and benzene : cyclohexane (9:1). Isotopes that were at least 95% pure were considered acceptable for use.

Experimental procedure

Rats were fasted for 12 hours and were then anesthetized lightly with ether and dosed by stomach tube with about 1.6 uCi of [1-^{3}H (N)] vitamin A and about 1.0 uCi of [4-^{14}C] vitamin D_3 dissolved in 0.1 ml of corn oil. Dosage was determined for each rat by weighing contents of syringes used for injection and by determination of radioactivity in weighed samples of the daily dosage solutions. Cold carrier was added so that equal amounts of about 12.5 ug of vitamins A and D were given.

Tail cups were placed on rats for the collection of feces since rats may consume as much as 50% of their own feces even when kept in wire screen bottom cages (*Nutr. Rev.*, 1962). Tail cups were constructed and attached as initially described by Barnes *et al.* (1957) and as modified by Kwong and Barnes (1979) except that it was necessary to wrap the plastic tail cups with wire screen to prevent the rat from chewing the plastic.

Rats were sacrificed by ether overdose 18 hours after dosing with the vitamins. Preliminary experiments had shown that more than 90% of the dose was out of the small intestine by 18 hours after dosing. The gastrointestinal tract was rapidly removed and sectioned into esophagus, stomach, small intestine, cecum and colon. The small intestine was further sectioned into five approximately equal segments. Contents of these segments were removed by a rinse of 2.5 ml of distilled water. It was determined experimentally that residual fluid was successfully removed by forcing air through the segment followed by manual compression of the segment. Therefore, water rinses were followed by two air rinses using a 10 ml syringe filled with air. The segments were then cleared of fat, blotted and weighed. Segment lengths were determined using a uniform weight (in this case, 2.7 mg) to standardize tension. The liver, kidney, and a sample of blood were also taken at sacrifice.

Isotope content of the esophagus, stomach, small intestine walls and rinses, plasma, liver, and kidney was determined by immediate solubilization of tissues taken at sacrifice in NCS tissue solubilizer (Amersham, Arlington Heights, IL) followed by liquid scintillation counting. Radioactivity in the cecum, colon, and feces was determined by total oxidation of triplicate aliquots of distilled water homogenates of the tissue in a tissue oxidizer followed by assay of radioactivity in a liquid scintillation counter. Tissues to be oxidized were kept frozen at −80°C until homogenization.

Determination of abosroption

The total percentage of the dose absorbed was determined by adding the percentage of the dose remaining in the gastrointestinal tract and in the intestinal rinses to the percentage of the dose in the feces collected during the 18 hours after dosing and subtracting this number from the total dose administered. Data were also expressed as percentage of total dose absorbed per 100 g of body weight, per 100 cm of small intestine, and per g of small intestine. Since the same amount was administered to each of the animals, these expressions of data allowed examination of any effect of differences in body weight, small intestinal weight, or small intestinal length on absorption.

Statistical treatment of data

Data were analyzed by a one way analysis of variance, followed by Kramer's modification of the Duncan's multiple range test at $p > 0.05$.

TABLE 1. THE EFFECT OF AGE ON THE BODY WEIGHTS AND ORGAN WEIGHTS OF FEMALE WISTAR RATS

Age (months)	Body weight (g) Mean ± SEM	Liver weight (g) Mean ± SEM	Kidney weight (g) Mean ± SEM	SI* weight (g) Mean ± SEM	SI length (cm) Mean ± SEM
6	267 ± 6[a] (11)**	7.2 ± 0.2[a] (11)	1.1 ± 0.1[a] (11)	4.8 ± 0.09[a] (11)	103.6 ± 1.7[a] (11)
12	297 ± 12[a] (13)	8.0 ± 0.3[a] (14)	1.2 ± 0.1[a] (14)	4.43 ± 0.13[a] (14)	111.1 ± 1.5[b] (14)
24	372 ± 20[b] (9)	11.0 ± 0.6[b] (9)	1.6 ± 0.1[b] (9)	5.34 ± 0.38[b] (8)	114.8 ± 1.9[b] (9)

*SI = small intestine.
**Number in parenthesis is *n*.
Means with different letters are significantly different at $p > 0.05$.

RESULTS

The 24 months old rats were significantly heavier and had significantly larger livers and kidneys than the 6 and 12 months old rats (Table 1). Small intestine weight was significantly greater in the 24 months old rats while small intestine length was significantly greater in both the 24 and 12 compared to the 6 months old rats.

The age of the animals did not have a significant effect on absorption of either vitamin A (Table 2) or vitamin D (Table 3) expressed as total percentage of the dose, total percentage of the dose absorbed per 100 g of body weight, percentage absorbed per 100 cm of small intestine, or as percentage absorbed per g of small intestine. The pattern of distribution of the dose of A and D also did not differ significantly as a function of age (Figs. 1 and 2).

DISCUSSION

This study examined total absorption of vitamins A and D under conditions as close as possible to the normal consumption and absorption of these vitamins by the rat. The techniques used offered several advantages over those of many *in vitro* and *in vivo*

TABLE 2. THE EFFECT OF AGE ON ABSORPTION OF VITAMIN A IN FEMALE, WISTAR RATS

Age (months)	Total % absorbed Mean ± SEM	% absorbed per 100 g body weight Mean ± SEM	% absorbed per 100 cm SI* Mean ± SEM	% absorbed Mean ± SEM
6	67.8 ± 4 (9)**	25.7 ± 1.7 (9)	65.8 ± 4.0 (9)	16.6 ± 1.3 (9)
12	63.8 ± 5 (10)	21.7 ± 2.0 (9)	53.9 ± 4.0 (8)	14.7 ± 1.0 (10)
24	74.6 ± 4 (7)	21.7 ± 2.0 (7)	65.9 ± 2.6 (7)	14.1 ± 1.3 (6)

*SI = small intestine.
**Number in parenthesis is *n*.
Differences were not significant at $p > 0.05$.

TABLE 3. THE EFFECT OF AGE ON ABSORPTION OF VITAMIN D IN FEMALE, WISTAR RATS

Age (months)	Total % abosrbed Mean ± SEM	% absorbed per 100 g body weight Mean ± SEM	% absorbed per 100 cm SI* Mean ± SEM	% absorbed g SI Mean ± SEM
6	52.1 ± 2.7 (8)**	19.5 ± 1.0 (8)	50.1 ± 2.4 (8)	12.3 ± 0.8 (8)
12	53.4 ± 4.7 (11)	17.3 ± 1.3 (9)	46.9 ± 3.6 (9)	12.1 ± 1.0 (11)
24	60.9 ± 5.5 (7)	16.5 ± 1.7 (7)	54.0 ± 5.0 (77)	11.1 ± 1.4 (6)

*SI = small intestine.
**Number in parenthesis is *n*.
Differences were not significant $p > 0.05$.

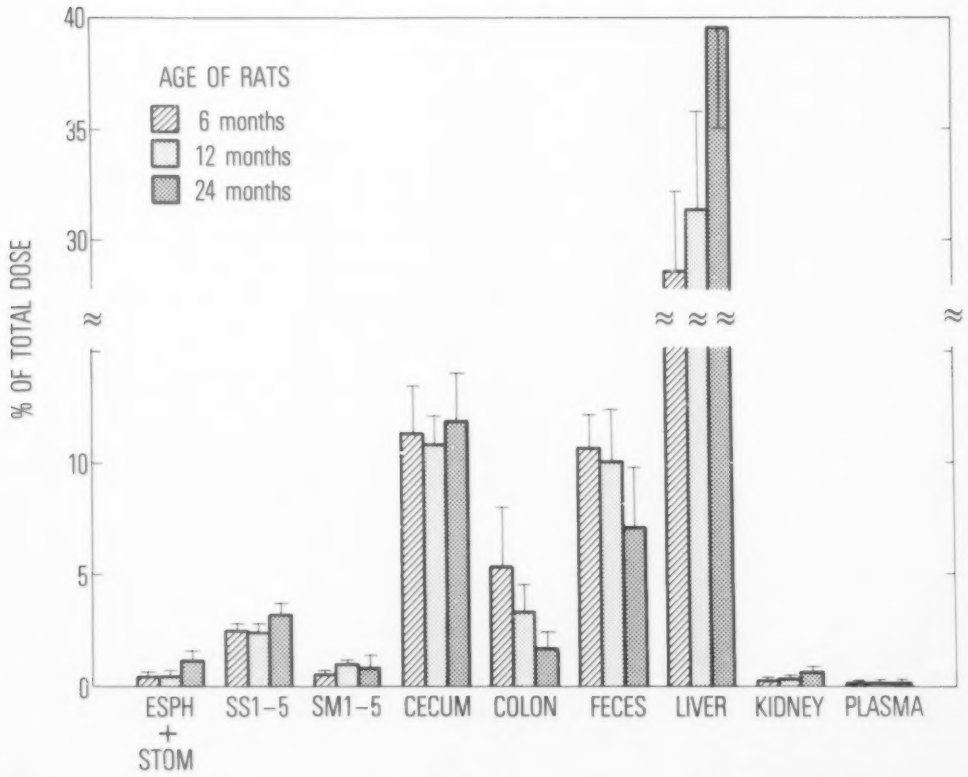

FIG. 1. Distribution of the administered dose of vitamin A among tissues of female Wistar rats. Vertical lines represent standard error for each mean value. ESPH + STOM is the isotope content of the entire esophagus and stomach; SSI-5 is the isotope content of the combined rinses for each of the intestinal segments; and SMI-5 is the isotope content of the segment walls. The % of the total dose in the liver and kidney are given for the total liver and for the right kidney. Plasma isotope content is given per ml of plasma.

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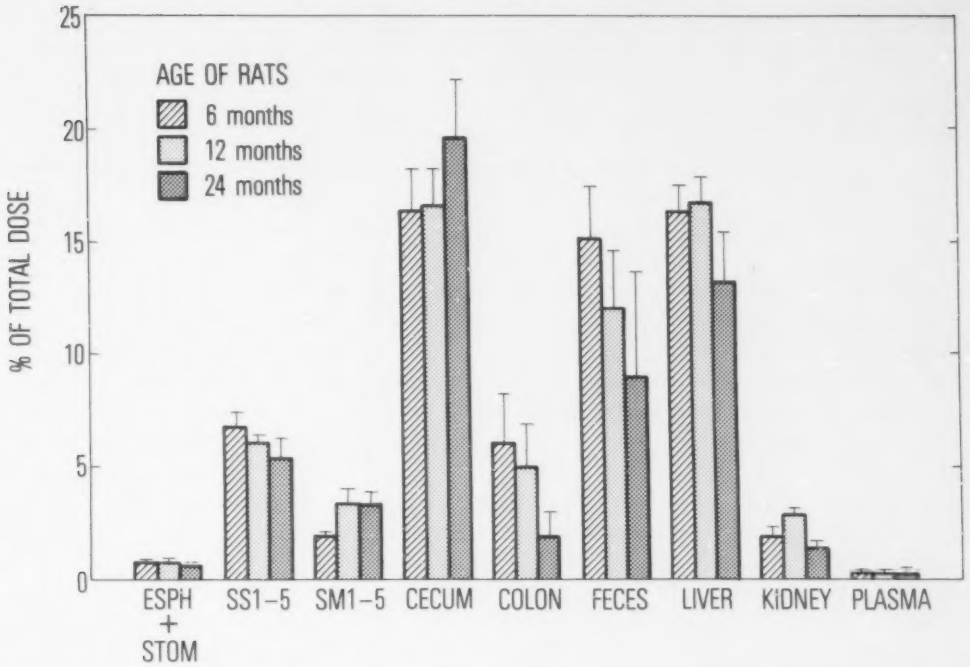

FIG. 2. Distribution of the administered dose of vitamin D among tissues of female Wistar rats. Vertical lines represent standard error for each mean value. ESPH + STOM is the isotope content of the entire esophagus and stomach; SS1-5 is the isotope content of the combined rinses for each of the intestinal segments; and SM1-5 is the isotope content of the segment walls. The % of the total dose in the liver and kidney are given for the total liver and for the right kidney. Plasma isotope content is given per ml of plasma.

methods used to study vitamin absorption. Rats were anesthetized only lightly for dosing and were awake during the period of absorption. The vitamins were given dissolved in corn oil which was similar to the manner in which these vitamins are presented to the gut. The method used in the present study avoids modification of the intestine or its blood supply that occurs in *in vitro* models and in many *in vivo* models. In addition, by examining absorption over an 18 hour period, a good approximation of the complete absorption of the vitamins was obtained.

Age was not a significant factor influencing the absorption of vitamins A and D in the present study. Hollander and Morgan (1979) studied absorption of vitamin A by cannulating the small intestines and administering similar amounts of vitamin A to 1.5, 12, 19, 25 and 39 months old male Sprague-Dawley Rats. The 12, 19, and 25 months old rats did not differ significantly in absorption of the dose, absorbing 28.1%, 29.3%, and 33.4% of the total dose respectively. The 1.5 months old rats absorbed less of the total dose (24.5%) and the 39 months old rats absorbed more (37.3%) than the 12, 19, and 25 months old rats. However, body weights of rats in Hollander's study ranged from a mean of 169 g in the 1.5 months old rats to 860 g in the 39 months old rats. When these large differences in body weights were taken into account by expressing Hollander's data as absorption per 100 g of body weight, absorption was 14.7%, 4.0%, 4.6%, 4.2%, and 4.2% of the total dose in the 1.5, 12, 19, 25 and 39 months old rats respectively. This method of

expression shows no differences in absorption among the four older age groups. Differences in vitamin absorption between the young growing rats and the four older groups may result from rapid growth in this group. Therefore, Hollander and Morgan's study agrees with the present study in finding no differences in absorption of vitamin A among adult rats.

In a study designed to examine vitamin D absorption in human subjects, Barragry *et al.* (1978) reported that plasma [^{3}H] vitamin D_3 response after oral ingestion of [^{3}H] vitamin D_3 was significantly lower in elderly females than in a group of young females. Plasma levels of vitamin D_3 and metabolites were monitored for only 6 hours following administration of the vitamin. Thus, differences between the two age groups could have been due to the time course of absorption and not due to quantitative differences in absorption. Indeed, Thompson and co-workers (1966) found that plasma radioactivity peaks 6 to 12 hours after administration of an oral dose of 0.1 to 1.0 mg of [^{3}H] vitamin D_3 to human subjects. Therefore, had Barragry and co-workers extended the time period of their study, they might have obtained a more complete estimate of total absorption. In the present study, absorption of doses of vitamins A and D was examined for the entire period of time during which absorption occurs. Age of the animals had no significant influence on absorption of vitamins A and D under these conditions.

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SUMMARY

The absorption of vitamins A and D was examined in 6, 12, and 24 months old female Wistar rats. Animals were dosed by stomach tube with radioactive forms of vitamins A and D and killed 18 hours later. Isotope in feces collected during this time and isotope remaining in the gastrointestinal tract were determined and subtracted from total dose to determine percentage of absorption. Under the conditions of this study, age had no significant effect on the absorption of vitamins A and D.

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Experimental Gerontology, Vol. 17, pp. 121–126, 1982
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THE INFLUENCE OF AGING ON INTESTINAL ABSORPTION OF VITAMIN B_{12} AND NIACIN IN RATS

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(*Received* 27 *June* 1981)

INTRODUCTION

LOWER SERUM levels of certain nutrients have been reported in older as compared to younger individuals in several studies (Barrows and Kokkonen, 1977; Fleming, 1981). These findings along with reports of increased generation time (Lesher *et al.* 1961; Thrasher and Greulich, 1965) among a decreased number of proliferative cells (Thrasher, 1971), decreased intestinal enzyme activity (Sayeed, 1967), and alterations in the intestinal microflora (Gorbach *et al.* 1967) suggest possible age-related changes in absorptive capacity of the gut. Data on changes in absorption as a function of age are limited and contradictory for some nutrients and non-existent for others. However, knowledge of changes in absorption as a function of age is essential for determining vitamin requirements of older adults.

The purpose of this study was to examine absorption of orally administered doses of B_{12} and niacin in unanesthetized 6, 12, and 24 month old rats.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats, 6, 12, and 24 months old, were born and reared in the Gerontology Research Center (GRC) animal colony. The rats were housed five to a 41 cm × 29 cm × 18 cm cage, maintained at a constant temperature of 72°F, and exposed to a 12 hour light/dark cycle. Rats were placed in the experimental animal rooms for 2 weeks prior to the experiment under conditions similar to those in the GRC colony except that they were housed individually. Two weeks of observations of body weights and general appearance helped to insure that only healthy rats were used in the experiments and gave the rats time to adapt to laboratory conditions prior to the experiment. Rats were fed Purina Rat Chow® *ad lib* throughout their life span.

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Isotopes

[Carboxyl-^{14}C] nicotinic acid with a specific activity of 448 uCi/mg (Amersham, Arlington Heights, IL) was checked for purity by thin layer chromatography on silica gel in a developing system of acetic acid: acetone: methanol: benzene (5: 5: 20: 70). The purity of the batches used was greater than 95%.

Cyano [G-^{3}H] cobalamin with a specific activity of 1.8 mCi/mg (Amersham, Arlington Heights, IL) was 94% pure as determined by dilution analysis according to manufacturer's specifications.

Experimental procedure

Rats were fasted for 12 hours and were then anesthetized lightly with ether and dosed by stomach tube with about 1.0 uCi (0.55ug) of ^{3}H-cyanocobalamin and about 1.0 uCi (2.0ug) of ^{14}C-nicotinic acid dissolved in 0.1 ml of H_2O. Exact dosage was determined for each rat by weighing contents of syringes used for injection and by determination of radioactivity of weighed samples of the dosing solutions used each day.

Tail cups were placed on the rats for collection of feces since rats may consume as much as 50% of their own feces even when kept on wire screen bottom cages (*Nutr. Rev.*, 1962). Tail cups were constructed and attached as initially described by Barnes *et al.* (1957) and as modified by Kwong and Barnes (1979) except that it was necessary to wrap the plastic tail cups with wire screen to prevent the rat from chewing the plastic.

Rats were sacrificed by ether overdose 16 hours after dosing with the vitamins. Preliminary experiments had shown that more than 90% of the dose had cleared the small intestine by 16 hours after dosing. The gastrointestinal tract was rapidly removed and sectioned into esophagus, stomach, small intestine, cecum, and colon. The small intestine was further sectioned into five approximately equal segments. Contents of these segments were removed by a rinse of 2.5 ml of distilled water. It was determined experimentally that residual fluid was successfully removed by forcing air through the segment followed by manual compression of the segment. Therefore, water rinses were followed by two air rinses using a 10 ml syringe filled with air. The segments were then cleared of fat, blotted and weighed. Segment lengths were measured after attaching a uniform weight (in this case, 2.7 mg) to standardize the tension of each segment. The liver, kidney, and a sample of blood were also taken at sacrifice.

Isotope content of the esophagus, stomach, small intestinal walls and rinses, plasma, liver, and kidney was determined by immediate solubilization of tissue taken at sacrifice in NCS tissue solubilizer (Amersham) followed by liquid scintillation counting. Radioactivity in the cecum, colon, and feces was determined by total oxidation of triplicate aliquots of distilled water homogenates of the tissue in a tissue oxidizer followed by assay of radioactivity in a liquid scintillation counter. Tissues to be oxidized were kept frozen at −80°C until homogenization.

Determination of absorption

The total percentage of the dose absorbed was determined by adding the percentage of the dose remaining in the gastrointestinal tract and in the intestinal rinses to the precentage of the dose in the feces collected during the 16 hours after dosing and subtracting this number from the total dose administered. Since the same amount was administered to each of the animals, this method of expression of data allowed examination of any effect of differences in body weight, small intestinal weight, or small intestinal length on absorption.

Statistical treatment of the data

Data were analyzed by a one way analysis of variance followed by Kramer's modification of the Duncan's multiple range test at $p > 0.05$.

RESULTS

Body weights, liver and kidney weights, and length and weight of the small intestine did not differ significantly among the three age groups (Table 1). Absorption of vitamin B_{12} and of niacin expressed as total percentage of the dose absorbed, percentage absorbed per 100 g body weight, percentage absorbed per 100 cm of small intestine, or percentage absorbed per g of small intestine did not differ significantly among the three groups (Tables 2,3). The pattern of distribution of the dose in the gastrointestinal tract, liver, kidney, or plasma also was not significantly influenced by the age of the animals (Figs. 1, 2).

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TABLE 1. ANIMAL WEIGHTS AND ORGAN WEIGHTS AND LENGTHS

Age (months)	Body weight (g) Mean ± SEM	Liver weight (g) Mean ± SEM	Kidney weight (g) Mean ± SEM	SI* weight (gm) Mean ± SEM	SI Length (cm) Mean ± SEM
6	337 ± 13 (10)**	9.0 ± 0.2 (11)	1.4 ± 0.1 (11)	5.4 ± 0.2 (11)	112 ± 1 (11)
12	345 ± 10 (13)	9.4 ± 0.5 (15)	1.3 ± 0.1 (15)	5.4 ± 0.8 (15)	109 ± 1 (15)
24	353 ±36 (8)	11.2 ± 0.6 (9)	1.5 ± 0.1 (9)	5.8 ± 0.2 (9)	116 ± 2 (9)

*SI = small intestine.
**Number in parentheses is *n*.
Differences were not significant at $p > 0.05$.

DISCUSSION

Malabsorption of vitamin B_{12} with age was suggested by an early study which reported a diminished response of serum B_{12} levels in old individuals following oral administration of 100 ug of B_{12} compared to the response of younger subjects (Chow, 1954). In rats, Draper and Lowe (1958) also reported an influence of age on absorption of B_{12}.

However, most reports indicate that there is no general impairment of absorption of vitamin B_{12} as a function of age. The Schilling test, which typically consists of administration of a test dose of approximately 2 ug of oral radioactive B_{12} followed by injection of a flushing dose of approximately 1 mg of unlabelled B_{12} within a few hours after which urine is collected for a 24 hour period, has been employed by several investigators who observed that age was not a significant factor in B_{12} absorption (Swendseid *et al.*, 1954; Chow *et al.*, 1956; Tauber *et al.*, 1957). More evidence for this view has come from human studies in which hepatic uptake of B_{12} was determined following oral administration of 0.5 ug radioactive B_{12} (Hyams 1964), from determination of the amount of B_{12} appearing in the feces after oral administration of 0.7 ug of ^{58}Co-labelled B_{12} (Hyams, 1964) and from observation of changes in serum levels of B_{12} after daily administration of oral doses of 20 ug B_{12} for 12 weeks to elderly subjects (Davis *et al.*, 1965).

Table 2. ABSORPTION OF B_{12}

Age (months)	Total % absorbed Mean ± SEM	% absorbed per 100 g body weight Mean ± SEM	% absorbed per 100 cm SI* Mean ± SEM	% absorbed per g of SI Mean ± SEM
6	19.6 ± 5.1 (8)**	7.0 ± 1.7 (7)	20.5 ± 4.4 (6)	3.5 ± 0.8 (7)
12	20.6 ± 1.8 (12)	6.0 ± 0.5 (11)	18.8 ± 1.5 (11)	3.1 ± 0.1 (9)
24	26.2 ± 7.9 (6)	7.0 ± 2.1 (6)	22.5 ± 5.7 (5)	4.5 ± 1.1 (6)

*SI = small intestine.
**Number in parentheses is *n*.

TABLE 3. ABSORPTION OF NIACIN

Age (months)	Total % absorbed Mean ± SEM	% absorbed per 100 g body weight Mean ± SEM	% absorbed per 100 cm SI* Mean ± SEM	% absorbed per g of SI Mean ± SEM
6	81.8 ± 1.8 (8)**	25 ± 1 (7)	73 ± 2 (7)	16 ± 1 (7)
12	82.6 ± 1.0 (12)	24 ± 1 (10)	74 ± 1 (10)	15 ± 1 (10)
24	80.1 ± 3.7 (6)	22 ± 2 (5)	68 ± 4 (5)	14 ± 1 (5)

*SI = small intestine.
**Number in parentheses is *n*.

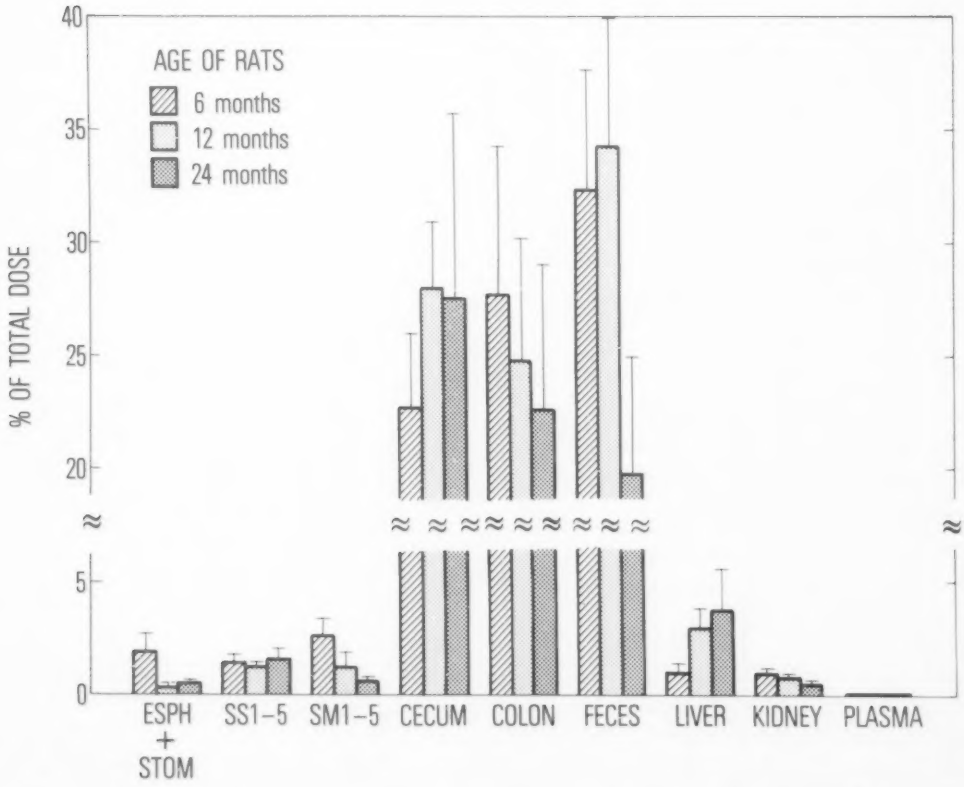

FIG. 1. Distribution of the administered dose of B_{12} among tissues of female Wistar rats. Vertical lines represent standard error for each mean value. ESPH + STOM is the isotope content of the entire esophagus and stomach; SSI-5 is the isotope content of the combined rinses for each of the intestinal segments; and SMI-5 is the isotope content of the segment walls. The % of the total dose in the liver and kidney are given for the total liver and for the right kidney. Plasma isotope content is given per ml of plasma.

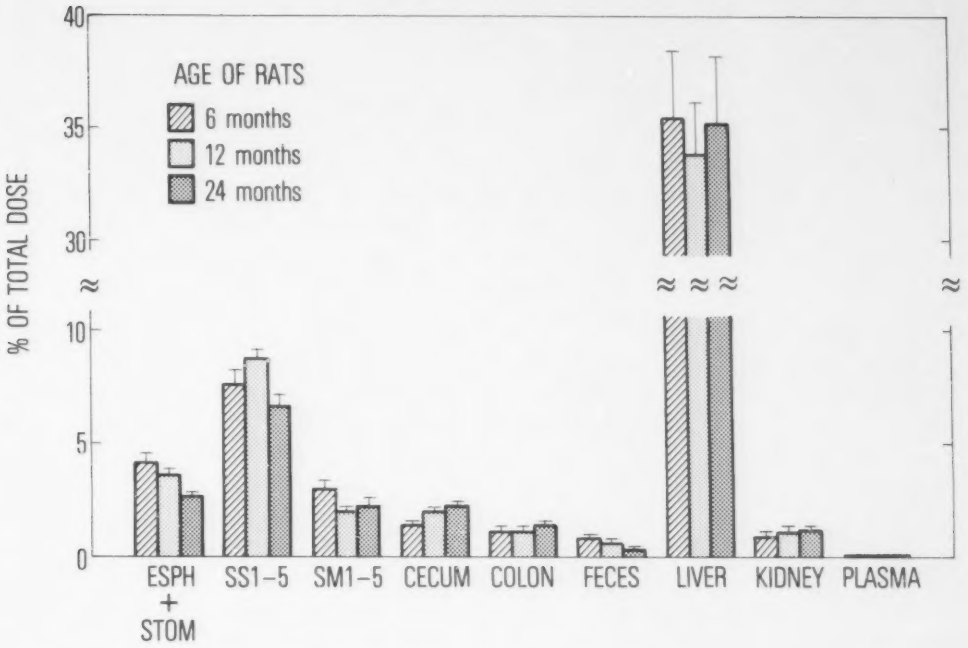

FIG. 2. Distribution of the administered dose of niacin among tissues of female Wistar rats. Vertical lines represent standard error for each mean value. ESPH + STOM is the isotope content of the entire esophagus and stomach; SSI-5 is the isotope content of the combined rinses for each of the intestinal segments; and SMI-5 is the isotope content of the segment walls. The % of the total dose in the liver and kidney are given for the total liver and for the right kidney. Plasma isotope content is given per ml of plasma.

Draper and Lowe examined cyanocobalamin absorption in 2.5, 8, 12, 16, 20, and 22 months old male Sprague-Dawley rats by administering ^{60}Co-cyanocobalamin by stomach tube and collecting feces for the following 48 hours. Absorption of the dose did not differ significantly among the 8, 12, 16 or 20 months old rats. Absorption of the vitamin was significantly less in the 2.5 months old growing animals and in the 22 months old rats compared to the other age groups. The 2.5 and 22 months old groups absorbed about 45% of the dose while absorption in the 12 months old group, for example, average 58% of the dose. A possible cause for differences in this study and Draper's could be the differences in dose size. Draper and Lowe administered 0.1 ug of ^{60}Co-cyanocobalamin while 0.5 ug ^{3}H-cyanocobalamin was given in the present study. Absorption of B_{12} is intrinsic factor dependent at lower concentrations, with passive absorptive processes assuming more importance at higher concentrations of the vitamin (Herbert *et al.*, 1964; Glass, 1963). However, Gaffney and co-workers examined the effect of age of human subjects on the percent of orally administered ^{60}Co-cyanocobalamin in amounts of 2, 8, 50, and 250 ug. There were no sytematic age differences in absorption at any of these dose levels (Gaffney *et al.*, 1959). Therefore, it is unlikely that differences between findings in Draper's study and the present study are entirely due to differences in dose size.

Absorption of B_{12} was highly variable particularly among the 6 and 24 months old rats

in the present study. However, absorption of B_{12} did not differ significantly among the three age groups.

There is no literature on niacin absorption as a function of age available for comparison to the present findings. However, niacin deficiency does not appear to be a serious problem among the healthy non-institutionalized elderly (Brin *et al.*, 1964; Harrill and Cervone, 1977) which suggests that intake and absorption are adequate.

There do not appear to be any age-related changes in absorption of B_{12} and niacin in rats. Therefore, diminished intestinal absorption is not a significant factor influencing nutritional status and requirements of these nutrients in healthy older animals.

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SUMMARY

The intestinal absorption of doses of vitaman B_{12} and of niacin was examined in 6, 12, and 24 months old female Wistar rats. Rats were dosed via stomach tube with radioactive forms of the vitamins and were killed 16 hours later. Percent of the dose remaining in the stomach and gastrointestinal tract and in the collected feces was determined. Absorption of the two vitamins was not influenced by the age of the animals.

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Experimental Gerontology, Vol. 17, pp. 127–132, 1982
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CHANGES WITH AGE IN THE AORTA OF MAN AND MOUSE

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(*Received* 27 *June* 1981)

ABSTRACT—Histologic and micrometric studies have been performed to examine changes with age in the aorta of man and mouse. The thickness of the aorta increased significantly with age while the number of nuclei in the aortic media decreased significantly in human beings as well as in mice.

INTRODUCTION

THOUGH THERE ARE many papers on senile changes of the aorta, there are still many questions to be clarified. Smith *et al.* (1951) reported progressive increase in the thickness of the aortic media of mice with advancing age. Movat *et al.* (1958) also observed progressive thickening of the human aorta with age that was more marked in the distal than the proximal portion. Cliff (1970) observed a two-fold increase in the thickness of the aortic media in aging rats.

Cliff (1970) also observed a 50% decrease in cellularity in the aortic media of aged rats. Kriessmann and Wagener (1970) reported a decrease in intimal muscle cells in human coronary arteries with age. Similarly, Nanda and Getty (1971) observed a decrease in smooth muscle cells in the tunica media of cerebral arteries in domestic pigs with increasing age.

The present study has been made to examine changes with age in the thickness of the aorta and cellularity of the aortic media in human beings and mice.

MATERIALS AND METHODS

The aortae of 100 human autopsy cases (accident cases, exclusively, ranging from 1 to 70 years of age) and 24 mice were used in the present study. The human post-mortem material was collected from the police-morgue of N.R.S. Medical College, Calcutta, India. The female mice of inbred 'Strain A' were procured from the mouse colony of Chittaranjan National Cancer Research Centre, Calcutta, India. These were fed with the standard laboratory diet of Hindustan Lever Ltd. At 5, 10, 15 and 20 months of age, six animals at each age level were killed. The aortae, human and mouse alike, were fixed in 10% neutral buffered formalin. Paraffin sections were cut at 4 μ and stained with haematoxylin and eosin.

The thickness of the aorta was measured with the help of an oculometer and a stage micrometer. The measurements were taken at five places from a single section and the average was calculated. The number of nuclei per unit area was counted in the aortic media.

OBSERVATIONS

Histologic and micrometric examination revealed the following changes with age in the aorta of human beings and mouse:

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TABLE 1. CHANGES WITH AGE IN THE THICKNESS OF THE HUMAN AORTA

Age group	Age in years	Number of subjects	Thickness of the aorta (intima + media) in mm
A	1-10	7	1.61 ± 0.1494
B	11-20	9	1.77 ± 0.0905
C	21-30	10	2.19 ± 0.0586
D	31-40	11	2.35 ± 0.0609
E	41-50	10	2.56 ± 0.0765
F	51-60	15	2.78 ± 0.0866
G	61-70	15	3.03 ± 0.0804

$P < 0.001$ = A and D, A and E, A and F, A and G, B and D, B and E, B and F, B and G, C and F, C and G, D and G, E and G.

$P < 0.01$ = A and C, B and C, C and E, D and F.

$P < 0.05$ = D and E, F and G.

NS = A and B, C and D, E and F.

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Human Aorta

Thickness of the aorta. The thickness of the aorta (intima + media) increased progressively with advancing age (Table 1; Fig. 1). The differences in the thickness of the aorta between the first decade and the fourth decade onwards were significant (<0.001). The differences between the second decade and the fourth decade onwards were also significant (<0.001).

Number of nuclei in the aortic media. A progressive decrease in the number of nuclei was observed with increasing age (Table 2; Fig. 2). The number of nuclei in the media was

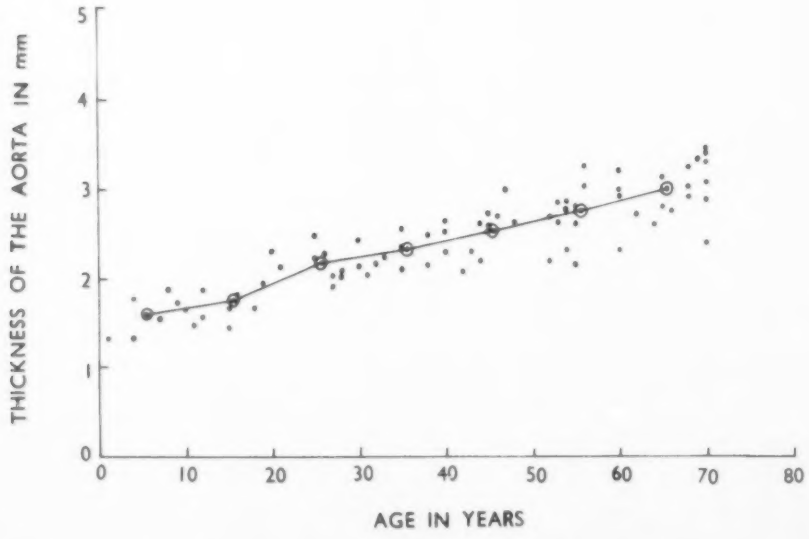

FIG. 1. Changes with age in the thickness of the human aorta.

TABLE 2. THE NUMBER OF NUCLEI IN THE MEDIA OF THE AORTA IN DIFFERENT AGE GROUPS OF HUMAN BEINGS

Age group	Age in years	Number of subjects	Number of nuclei in the aortic media per 70540 μm^2 area
A	1–10	7	178.86 ± 9.92
B	11–20	9	152.78 ± 7.06
C	21–30	10	116.40 ± 7.94
D	31–40	11	102.91 ± 5.46
E	41–50	10	100.80 ± 5.29
F	51–60	15	92.67 ± 3.67
G	61–70	15	76.47 ± 3.92

$P < 0.001$ = A and C, A and D, A and E, A and F, A and G, B and D, B and E, B and F, B and G, C and G, D and G, E and G, F and G.
$P < 0.01$ = B and C.
$P < 0.05$ = C and F.
NS = A and B, C and D, C and E, D and E, D and F, E and F.

reduced to more than a half in the seventh decade than that of the first decade. The differences in the number of nuclei between the first decade and the third decade onwards were significant (<0.001). The differences were also significant between the second decade and the fourth decade onwards (<0.001).

Mouse Aorta

Thickness of the aorta. A progressive increase in the thickness of the aorta (intima + media) was observed with advancing age (Table 3; Fig. 3). The difference in the thickness of the aorta of mice aged between 5 and 20 months was significant (<0.01). The differences in the thickness of the aorta were also significant between 5 and 15 months, and 10 and 20-months-old animals (<0.05).

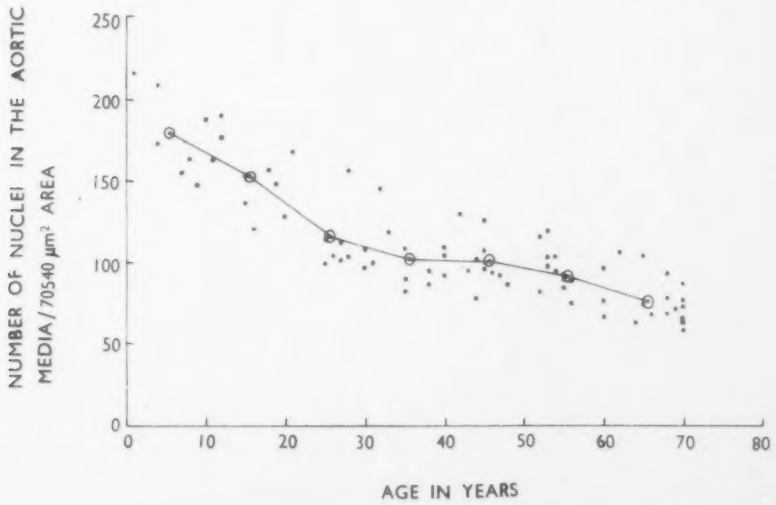

FIG. 2. Changes with aging in the number of nuclei in the human aortic media.

TABLE 3. CHANGES WITH AGE IN THE THICKNESS OF THE AORTA OF THE MOUSE

Age group	Age	Number of animals	Thickness of the aorta (intima + media) in μm
A	5 months	6	59.11 ± 3.17
B	10 months	6	62.60 ± 2.48
C	15 months	6	68.91 ± 2.16
D	20 months	6	71.66 ± 2.01

$P < 0.01$ = A and D.
$P < 0.05$ = A and C, B and D.
NS = A and B, B and C, C and D.

Number of nuclei in the media of the aorta. The number of nuclei in the aortic media was found to decrease with increasing age (Table 4; Fig. 4). The differences in the number of nuclei between 5 and 15 months, 5 and 20 months, and 10 and 20-months-old animals were significant (<0.01).

DISCUSSION

The thickness of the aorta has been reported to increase with age in mice (Smith *et al.*, 1951), human beings (Movat *et al.*, 1958) and rats (Cliff, 1970). However, neither did they make their observations in different age groups nor did they analyse their findings statistically. The present study, based on the changes in different age groups, revealed a

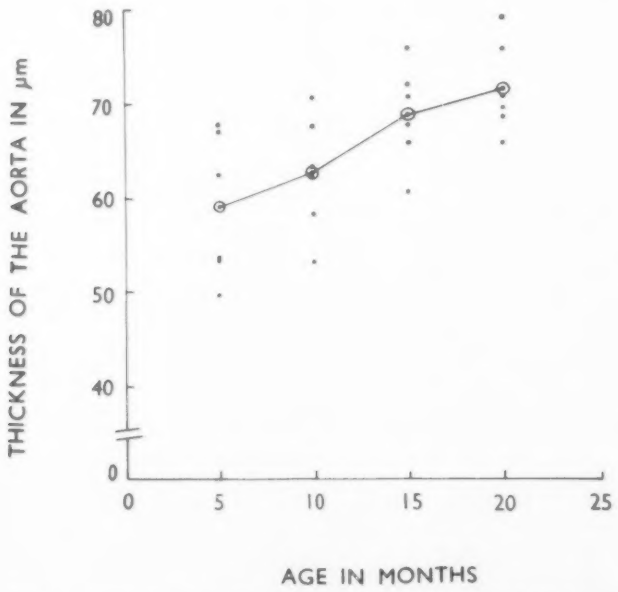

FIG. 3. Changes with age in the thickness of the aorta of the mouse.

TABLE 4. THE NUMBER OF NUCLEI IN THE MEDIA OF THE AORTA IN DIFFERENT AGE GROUPS OF MICE

Age group	Age	Number of animals	Number of nuclei in the aortic media per 1000 μm^2 area
A	5 months	6	222.36 ± 6.92
B	10 months	6	214.54 ± 6.70
C	15 months	6	187.74 ± 5.70
D	20 months	6	183.34 ± 5.82

$P < 0.01$ = A and C, A and D, B and D.
$P < 0.05$ = B and C.
NS = A and B, C and D.

significant increase in the thickness of the aorta of human beings and mice with the advancement of age (Tables 1, 3; Figs. 1, 3).

Cliff (1970) demonstrated a decrease in the number of cells in the tunica media of aged rats. No significant report exists, on the number of nuclei in the aortic media in different age groups of human beings and mice. The present study, however, showed a significant decrease in the number of nuclei in the aortic media with advancing age (Tables 2, 4; Figs. 2, 4). This observation may be corroborated by the findings of Stein *et al.* (1969) who observed a decrease of biochemically-determined DNA content in the aortas of aged rats.

Spraragen *et al.* (1962) believed that the decrease in cellular content of the aortic media was due to an increase in the thickness of the aorta in the absence of cellular hyperplasia.

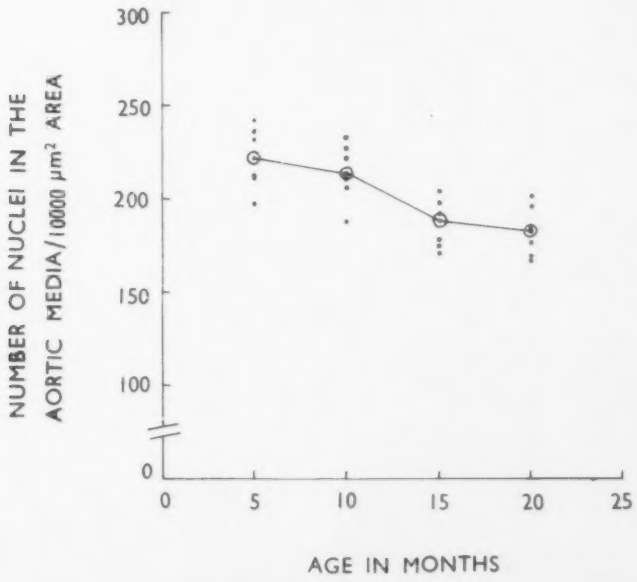

FIG. 4. Changes with aging in the number of nuclei in the aortic media of the mouse.

Tauchi (1961) considered a decrese in the number of cells to be the main characteristic of senile change.

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Experimental Gerontology, Vol. 17, pp. 133–138, 1982
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AGE-RELATED VASOPRESSIN CHANGES IN RAT PLASMA AND THE HYPOTHALAMO-HYPOPHYSEAL SYSTEM

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(*Received* 27 *June* 1981)

INTRODUCTION

EVIDENCE FOR an age-related decline in vasopression (VP) secretion in rats has been mainly indirect. In old-intact rats the outward shift of water and electrolytes from the cells is similar to that observed in young rats with experimentally induced diabetes insipidus (Friedman *et al.*, 1963a, 1963b).

Age-related changes in the physiology of VP have been studied in various ways. Turkington and Everitt (1976) demonstrated significant decreases in plasma VP concentration and urinary excretion in old (up to 30 months) rats. The responsivity of the central osmoreceptor mechanism was found to be refractory to intracarotid injection of hypertonic saline in 20-month-old rats. This was interpreted as evidence for a decline in neurohypophyseal function with aging (Friedman *et al.* 1956). The authors concluded that the NH in old Wistar rats has an impaired ability to secrete VP.

The studies of the effect of age on VP content in the neurohypophysis (NH) are mainly based on the histological observation of the neurosecretory material (NSM), yielding incomplete and conflicting data. Rodeck *et al.* (1960) did not detect any difference in the amount of NSM between young (3–6 months) and old (12–24 months) rats. However, in the senile (approx. 36 months), the amount of NSM was decreased. These authors further showed, using VP pressor assay, that the young and old rats have a similar content of VP/NH. Danihue (1965) found less NSM in 30 months than in 10 months old rats and he interpreted this as an age-related reduction in the synthesis and storage of neurohypophyseal hormones. However, using the same techniques in a study spanning 19 months, Morrison and Staroscik (1969) demonstrated an increase in NSM with aging.

The effect of aging on VP content of the rat hypothalamus (HT) is not well known. Rodeck *et al.* (1960) demonstrated no difference in the amount of NSM in the HT of rats

Supported in part by NIH Grant 2 SO7 RR05393 (Biomedical Research Support Grant).

12–14 months and 6 months old. However, in the senile (approx. 36 months) rats, the NSM was scanty. To our knowledge, there are no available data based on direct measurement of VP in the rat HT that support these histological findings.

To verify the effect of aging on the VP system, we measured (by radioimmunoassay, RIA) VP content in HT and NH and plasma VP concentration in old, adult and young rats. Halothane anesthesia, a stimulus known to increase plasma VP concentration in the rat (Aziz & Forsling, 1979) was used to study the age-related responsivity of the hypothalamo-neurohypophyseal system.

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METHODS AND PROCEDURE

Male Sprague-Dawley rats (Blue Spruce Farm) were used. *Old* animals were purchased as retired breeders (9 months) and aged in the animal quarter up to 30 months. *Adult* animals were also purchased as retired breeders and were kept 3 months to reach the age of 12 months. *Young* animals were purchased at the age of 2 months and kept one month prior to the study. All animals were kept in the same room under the same conditions: constant temperature of 22.5 ± 0.4°C, 12:12 light-dark cycle and a rat Purina Chow® diet and tap water *ad libitum*.

The experimental rats were anesthetized with 1 MAC (minimal alveolar concentration) to obtain the same depth of anesthesia in each age group. The young received 1.0%, the adult 1.2% and the old 1.4% halothane-O_2 delivered at 10 l/min flow through a Fluotec vaporizer Mark 1 into a 9 liter chamber. The chamber halothane concentrations were monitored by a calibrated Norcotest. After one hour of anesthesia, the rats were decapitated as well as the control animals and trunk blood was collected into heparinized dishes. The autopsy was performed, spleen removed and weighed, and hematocrit measured in all studied animals. Plasma was frozen for VP extraction done within 10 days. The NH including pars intermedia and the HT tissue block (approximately 2 mm from the midline bilaterally at the base of the brain and bound by chiasma opticum anteriorly and the corpora mamillaria posteriorly), according to Holecek *et al.*, (1962) were quickly removed. This was always done by one of us (VKZ) to standardize the technique. Larger block tissue was dissected in adult and old (100–150 mg) than in young (80–100 mg). Tissues were weighed and dried in acetone for 2 days.

VP extraction

Plasma VP was extracted with cold acetone and petroleum ether (Robertson *et al.*, 1973) and kept frozen until radioimmunoassay. Duplicate 0.4 ml aliquots of the extract were assayed in each experiment. Dried HT and NH were homogenized with 2 ml of 0.25% acetic acid, boiled for 2 minutes, chilled on ice and centrifuged. The supernatant was mixed with drop of glacial acetic acid to keep pH below 3.0 and kept at 4°C until the assay.

VP measurement

An improved double-antibody radioimmunoassay (RIA) developed by Wu and Rockey (1969) was used to measure VP from plasma, NH and HT. In this system, different antiserum than the originally described was used at a final dilution of 1:70,000. Samples and calibration curve in duplicates were incubated for 3 days at 4°C and another 2 days after adding second antibody. Radioactivity in each sample was measured as "total" and "precipitate" and calibration curve was constructed as % of precipitate/total and the concentration of unknown samples was read from the calibration curve. Synthetic arginine vasopressin (sAVP, Organon, Lot #92291, 292 IU/mg) was used for establishing the standard curve and for labeling with Na ^{125}I. This RIA is capable of detecting 0.29 μU (1 pg) VP/sample as 10% displacement from zero. Within-assay and between-assay variances were 10–13 and 20% respectively.

This antiserum provides full cross reactivity with lysine vasopressin (Sigma), while very little with oxytocin (Sigma, <0.001%), vasotocin (Sigma, <0.001%) and angiotensin II (Beckman, <0.00001%).

Plasma samples were corrected for extraction recovery (75%) and expressed as μU/ml (1 pg = 0.29 μU). All results are presented as mean ± *S.E.* One way analysis of variance was used to analyze the data.

RESULTS

All animals had normal hematocrit (45–55%) and no animal showed enlarged-swollen spleen reflecting infectious disease. The results are summarized in Table 1.

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TABLE 1. THE EFFECT OF HALOTHANE ANESTHESIA ON VASOPRESSION (VP) LEVELS IN PLASMA, NEUROHYPOPHYSIS AND HYPOTHALAMUS IN YOUNG, ADULT, AND OLD RATS

	Young (3 months)		Adult (12 months)		Old (30 months)	
	Control	Halothane	Control	Halothane	Control	Halothane
No. of rats	16	16	10	10	6	6
Body weight (g)	266 ± 6	268 ± 7	477 ± 15[a]	476 ± 10[a]	518 ± 15[a]	519 ± 7[a]
Plasma VP (μU/ml)	1.2 ± 0.3	3.0 ± 0.5[d]	0.9 ± 0.5	2.4 ± 0.6	N.D.*	0.9 ± 0.4
Neurohypophysis						
mU/gland	209 ± 20	212 ± 21	318 ± 35[b]	304 ± 24[b]	516 ± 53[a]	545 ± 57[a]
mU/mg wet tissue	135 ± 15	141 ± 13	136 ± 16	149 ± 18	181 ± 14	165 ± 16
Hypothalamus						
mU/gland	11.3 ± 0.7	13.6 ± 1.1	12.6 ± 1.6	14.5 ± 1.5	5.1 ± 0.5[c]	4.8 ± 0.4[c]

*Not detectable.
[a] $p < 0.01$, compared to young group.
[b] $p < 0.05$, compared to young group.
[c] $p < 0.01$, compared to young and/or adult group.
[d] $p < 0.01$, compared to control group.

Plasma VP concentration

The measured values were similar in the young and adult control rats (1.2 ± 0.3 and 0.9 ± 0.5 μU/ml) while in the old animals, it was undetectable (<0.29 μU/0.4 ml).

Halothane anesthesia increased plasma VP concentration significantly in the young (3.0 ± 0.5 μU/ml; $p < 0.01$) but not in the adult rats (2.4 ± 0.6 μU/ml; $p > 0.05$). In the old rats, undetectable plasma VP concentration was elevated to a value of intact adult rats (0.9 ± 0.4 μU/ml).

VP content in the neurohypophysis

Total VP content in the NH was lowest in the young (209 ± 20 mU/NH), higher in the adult (318 ± 35 mU/NH; $p < 0.05$) and the highest in the old rats (516 ± 53 mU/NH; $p < 0.01$).

However, there was no difference when the results were expressed as mU/mg tissue (135 ± 15 mU/mg in the young, 136 ± 16 mU/mg in the adult and 181 ± 14 mU/mg in the old rats).

Halothane anesthesia did not change the VP contents of the NH in either group.

VP content in the hypothalamus

The measured values were similar in the young and adult rats (11.3 ± 0.7 and 12.6 ± 1.6 mU/HT) while in the old it was significantly decreased compared to both groups (5.2 ± 0.5 mU/HT; $p < 0.01$).

Halothane anesthesia did not change this pattern.

DISCUSSION

We have shown that the total amount of VP in the rat NH depends on glandular size and does, therefore, increase with age. However, when expressed as mU/mg wet tissue, VP content does not change between the age of 3 and 30 months. This clearly demonstrates that the storage of VP in NH is not impaired by aging. Our data of VP content in the NH from RIA can be compared with those from the pressor bioassay of Rodeck *et al.* (1960). They also found no significant difference between young and old, when VP content was expressed as mU/mg wet tissue. Contrary to our findings they also found no significant difference when VP content was expressed as mU/NH (941 ± 81 and 1100 ± 96 mU/NH) in young and old respectively. It is possible that the authors might have masked age-related difference by using rats of mixed age (3–6 months in the young group and from 12–24 months in the old group), while we used age groups of 3, 12, and 30 months. In addition, they used rats of both the Dusseldorf and Bristol strains while we used only Sprague-Dawley rats. This difference, in addition to the fact that we used RIA instead of a pressor bioassay, may explain the much lower VP content found in our study (209 ± 20, 318 ± 35, and 516 ± 53 mU/gland in young, adult, and old, respectively). Although Rodeck's group showed that the amount of NSM in NH was decreased in senile (approx. 36 months) rats, they did not specifically measure VP conent. Since our oldest group of rats was only 30 months, it is possible that in the older rat, we may have found that the VP content in NH was reduced.

Our results are not in agreement with those of Dunihue (1965) who found a sharp reduction in the amount of NSM in the NH of 30-month-old compared to 10-month-old Wistar rats. However, Morrison and Starscik (1964) also using Wistar rats reported greatly increased amounts of NSM in age up to 19 months supporting our data.

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Our results, show an age-dependent decline in the VP content of the HT. In the 30-month-old rats the VP content is less than half of that in the young and adult rats. These data are in agreement with those of Rodeck *et al.* (1960), who found decreased amounts of the NSM in the HT of 36-month-old rats, but not in those 12–24 months of age. We did not study 24-month rats specifically, but according to both their results and ours, one can suggest that the VP content of the HT begins to decline between 24 and 30 months of age. Additional studies are needed to verify this point.

The observation that VP storage in the NH is not diminished in the old rats whereas the VP content of the HT is dramatically reduced may reflect decreased synthesis or it may reflect increased rate of transport and/or increased storage capacity of the neurohypophysis.

The plasma VP concentrations in young and adult rats showed no difference. However, in old rats plasma VP was undetectable (<0.29 μU/0.4 ml), suggesting either reduced VP release from NH or increased degradation and inactivation. Turkington and Everitt (1976) found lower plasma VP concentration in old (23 and 30 months) than in young (6 and 13 months) Wistar male rats. The plasma VP concentration in our study (mean 1.2 μU/ml in the young vs <0.29 μU/0.4ml in the old) was much lower than that reported in their study (mean 5.0 and 5.5 μU/ml in the young vs 3.0 and 3.5 μU/ml in the old). There may be several reasons for these differences. Turkington and Everitt used Wistar rats and measured VP by bioassay while we used Sprague-Dawley rats and measured VP by radioimmunoassay. In addition, the season in which the experiments were carried out may have some bearing in the basal plasma VP concentration. Our experiments were performed in winter and we have shown previously that plasma VP concentration in young rats is much lower in the winter than in the summer season (Zbuzek & Wu, 1979). Whether such a seasonal pattern persists during aging is unknown. If it does, it ccould explain why the plasma VP concentration was undetectable in our old rats. Additional study of age-related plasma VP concentrations in the summertime may provide further clarification.

Halothane anesthesia was delivered in 1 MAC, which represents the depth of anesthesia under which 50% of rats in each group respond to tail pinching by movement. Old rats require more halothane (1.4%) than adult (1.2%) or young (1.0%) to obtain the same depth of anesthesia because older rats have more fatty tissue which dissolves halothane. Under these conditions, anesthesia should be an equal stimulus for each age group, but the plasma VP concentration increased only significantly in the young but not in the adult rats due to a large scatter. We have also observed in young rats that the plasma VP concentration in response to halothane anesthesia is greater in summer than in winter (unpublished observations). In our old rats, however, halothane anesthesia elevated the plasma VP concentration from undetectable levels to those measured in the young and adult control rats. The actual plasma VP concentration in old rats is unknown (<0.29 μU/0.4 ml) and therefore its elevation up to 0.9 μU/ml is difficult to analyze satisfactorily. Thus we interpret it, that the capacity of the NH to release VP in response to halothane anesthesia is reduced but not completely impaired by aging.

SUMMARY

Immunoreactive vasopressin (VP) was measured in the plasma, neurohypophysis (NH) and hypothalamus (HT) of young (3 months), adult (12 months), and old (30 months) Sprague-Dawley male rats, both intact and anesthetized with halothane.

VP content in the HT of the old rats was about half that of young and adult rats.

Total VP content in the NH increased with age, reflecting large glandular size in the old rats. However, VP content was similar in all three groups, when expressed per mg of wet tissue.

Plasma VP concentration was undetectable in the old intact rats and it rose to the levels of intact young or adult rats during halothane anesthesia. The anesthesia-related increase in plasma VP concentration was statistically significant in young but not in adult rats.

These results suggest that in the intact rats during aging: (1) the synthesis of VP in the HT may be reduced; (2) the storage of VP in the NH is not impaired; and (3) the undetectable plasma VP concentration may reflect either reduced VP release or increased degradation.

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Experimental Gerontology, Vol. 17, pp. 139–143, 1982
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0531-5565/82/020139-05$03.00/0
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THE EFFECT OF AGE ON BODY ENERGY CONTENT OF THE ANNUAL CYPRINODONT FISH, *NOTHOBRANCHIUS GUENTHERI*

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(*Received* 1 *July* 1981)

INTRODUCTION

RECENTLY THERE has been a growing interest in the use of the annual fish, panchax, in experimental aging studies. The relative value of using fish in aging research has been discussed by Woodhead (1978), and various researchers are beginning to explore this approach to gerontological research (*e.g.*, Walford and Liu, 1965; Liu and Walford, 1966, 1969, 1970; Cannon and Davidson, 1978; Markofsky *et al.*, 1972, 1973, 1976, 1979). Of the wide variety of annual fish available, the *Nothobranchius guentheri* (family *Cyprinodontidae*) appears to be the easiest to maintain in the laboratory with a minimum of husbandry problems. This fish is a mud spawner with a normal lifespan in captivity of 12 to 18 months. Their origin is East Africa where they have evolved in an environment having an annual rainy and dry season. Because they cannot normally survive the dry season, their lifespan has been scaled to approximately one year.

In the study reported here the energy content of the complete fish body was measured in a bomb calorimeter and correlated *vs.* fish age in an attempt to measure collagen crosslinking energetics. However, a slight decrease in body fat content with increasing age (Markofsky, 1976) apparently masked the crosslinking energy storage effects.

MATERIALS AND METHODS

Male and female *Nothobranchius guentheri* fish populations of various young ages were purchases commercially.* Several lots of 50 or more were purchased over a period of 2 years at ages between fry and 2 months. These fish were then maintained in laboratory facilities in unheated tanks at a density of approximately 1 fish/1 in 10 and 15 gallon aquaria. Young fish were fed live brine shrimp and then switched to TetraMin® from an automatic feeder. Freeze dried brine shrimp were mixed into their diets once or twice a week. The room temperature was maintained between 20 and 23°C during the entire experiment. Groups of 5 males and 5 females were culled from the stock at monthly intervals and dried in a vacuum oven at 40°C for 24 hours. They were then sealed in airtight capsules and stored at room temperature for later bomb calorimetry testing.

*All fish in this study were purchased from Mr. Ed Warner, 507 John Street, Rockford, Illinois.

A Parr model 1241 Automatic Adiabatic Bomb Calorimeter equipped with a model 1245 semimicro bomb system was used for testing the dried fish. This system is capable of testing samples from 25 to 200 milligrams liberating energies up to 1200 calories. The dried fish were first pelletized and then burned individually, except for the 1 month old fry which were pelletized in groups of 3 or 4 per test. Calorimeter data were analyzed on a computer using custom written software. The calorimeter system was calibrated with commercially available benzoic acid calorific standards. The bomb constant was determined from the average of 10 benzoic acid calibration tests and the standard deviation of the 10 tests was 17 cal/g.

RESULTS

Table 1 presents the results of body energy content measurements made with the bomb calorimeter on 43 (20 M, 12 F, 11 unsexed) *Nothobranchius guentheri* of various ages. The energy values are per gram of *dry* whole body weight and they show a significant decrease with increasing age. The larger standard error values for females may be due to variations in egg contents among the females.

DISCUSSION

It is now clear that in the biological world increasing age produces increasing molecular crosslinking among protein macromolecules (Eyre, 1980; Goosey and Zigler, 1978; Panigrahy and Patnaik, 1976; Deardin and Odusina, 1973; Stenzelet *et al.*, 1974; Sinex, 1964). Walford *et al.* (1969) have shown that the soluble/insoluble (*i.e.*, uncrosslinked/crosslinked) collagen ratio decreases with increasing age in the body of the annual fish *Cynolebias bellottii*. Recently, however, Cannon and Davison (1978) reported the negative results of a similar soluble/insoluble collagen ratio study on the skin of *N. guentheri*.

Since the chemistry of living biological systems obeys the classical laws of chemical reactions, then each biochemical reaction that occurs must be accompanied by a decrease in the (Gibbs) free energy of the system. Consequently, the net effect of biochemical reactions producing molecular crosslinking will be to decrease the free energy of the biological system. Since a bomb calorimeter measures the total molecular energy content of a sample, then all other things remaining equal, the effect of increasing molecular crosslinking with increasing age should appear as a decrease in body energy content per unit weight with increasing age. Bomb calorimetry has been used for some time in the field of synthetic polymers to determine the free energy decrease in polymerization reactions (*e.g.*, see Dainton and Ivin, 1950; Joshi and Zwolinski, 1967; Sawada, 1976). Therefore, it should

TABLE 1. SPECIFIC BODY ENERGY CONTENT *vs.* age of *N. Guentheri*

Group Age (Months)	Sex (Number)	Mean Energy Value (cal/g)*	± Standard Error** (cal/g)
1.0	mixed (11)	4496	155
2.25	M (5)	4579	81
2.25	F (4)	4473	195
3.0	M (5)	4375	68
4.0	M (1)	4010	–
6.0	M (8)	4047	51
6.0	F (3)	4866	401
11.0	M (1)	3800	–

*Dry weight.

**Standard Error – (Standard Deviation)/ $\sqrt{n}$.

be directly applicable to the biological use described here since macromolecular crosslinking is morphologically identical to polymerization.

Let Γ be the total energy decrease (deficit) within a living system over some time span due to molecular crosslinking. Then we can write:

$$\Gamma = \gamma mn \tag{1}$$

where γ = energy deficit per crosslinked bond,
m = number of crosslinks per macromolecules,
n = total number of crosslinked macromolecules in the system.

Unfortunately, the crosslinking chemistry of protein macromolecules is still largely unknown (Eyre, 1980). Collagen chemistry is currently the most completely understood with typical crosslinks arising from the condensation of aldehydic groups or of an aldehyde and amino group. Collagen is a fibrous protein whose amino acid residues are about 33% glycine, 21% proline and hydroxyproline, and 11% alanine. Collagen is the most abundant protein in living systems, often comprising one-quarter to one-third of the total system protein. Tropocollagen is the basic molecular subunit of collagen. It contains 3 polypeptide chains wound together in a triple helix to form a rigid rod 2800 Å long, 15 Å in diameter, with a total molecular weight of 300,000. The tropocollagen rod structure is stabilized with hydrogen bonding and crosslinking between the component polypeptide chains. Collagen polypeptide chains with no crosslinks are called α chains. Two α chains crosslinked together is called β collagen, and when all three chains are crosslniked it is known as γ collagen. Bailey (1970) has studied the nature of the crosslinking in the collagen of various fishes and concludes that collagen crosslinking is fundamental and independent of different morphological fiber arrangements.

Though a variety of crosslinking agents may be involved in biological aging, for purposes of illustration here we will focus on only two different types. First we consider a simply aldehyde covalent crosslink between two α collagen polypeptide chains.

$$2(\alpha - \mathrm{H}) + \underset{\mathrm{H}}{\overset{\mathrm{H}}{\mathrm{C}}} = \mathrm{O} \rightarrow \alpha - \underset{\mathrm{H}}{\overset{\mathrm{H}}{\mathrm{C}}} - \alpha + \mathrm{H_2O} \tag{2}$$

In equation (2) we have used formaldehyde to produce a methylene crosslink bridge between two α chains. A bond energy calculation for this reaction gives a net crosslinking energy deficit of 13 Kcal/mole. Consequently, the energy deficit per crosslink, γ, is given by:

$$\gamma = \frac{13}{A} \text{ Kcal/crosslink} \tag{3}$$

where A is Avogadro's number (6.02×10^{23} molecules/mole). A simple peptide bond crosslink can be modeled as follows:

$$2(\mathrm{HO} - \overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}} - \alpha - \overset{\mathrm{H}}{\overset{|}{\mathrm{N}}} - \mathrm{H}) \rightarrow \mathrm{HO} - \overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}} - \alpha - \overset{\mathrm{H}}{\overset{|}{\mathrm{N}}} - \overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}} - \alpha - \overset{\mathrm{H}}{\overset{|}{\mathrm{N}}} - \mathrm{H} + \mathrm{H_2O} \tag{4}$$

A bond energy calculation for the reaction of equation (4) gives a net energy deficit of 5 kcal/mole, and for this reaction:

$$\gamma = \frac{5}{A} \text{ Kcal/crosslink} \tag{5}$$

For purposes of calculation we will assume an average value of γ of 10/A Kcal/crosslink.

Let W be the dry weight of the biological system, and assume that it is 80% collagen. Then we can estimate the maximum total number of crosslinked collagen chains in the system, n, as:

$$n = 0.8\, W \left(\frac{A}{M}\right) \text{ chains} \tag{6}$$

where A = Avogadro's number, and M is the α chain molecular weight (10^5).

Finally, we must estimate the maximum number of crosslinks possible per α chain. We assume crosslink sites are readily available along the entire length of the chain. Atkins (1977) has introduced what he calls a "test tube brush" model of a proteoglycanhyaluronic acid complex and proposes that tropocollagen fibrils could be crosslinked with such a proteoglycan matrix. If one considers each glycine within the α chain as a possible peptide crosslink site, then there are about 330 such sites per α chain. It does not seem unreasonable to consider as many as 500 possible crosslinking sites of all types on each α chain which will allow crosslinking both within and between tropocollagen fibrils. Combining m = 500 with equations (5) and (6) into equation (1) gives:

$$\frac{\Gamma}{W} = 40 \text{ cal/(g dry Wt)} \tag{7}$$

Thus we should see a maximum energy deficit in the bomb calorimetry data of about 40 cal/g dry Wt for age induced crosslinking. Table 1 shows a much larger decrease in body energy content with increasing age.

Markofsky (1976) has studied the body composition of *N. guehtheri* at various points in their lifespan using both longitudinal and cross sectional observations. His data indicate that the total body water content is a constant fraction (78 to 81%) of the fat-free body weight (FFB) independent of age. The protein fraction of the FFB increases between 3 and 4.4 months, remains constant until 12.4 months, then declines slowly. He found the whole body fat content difficult to measure accurately, but from the cross-sectional observations it appeared to reach a maximum of 3.38 ± 0.19% at 4.4 months of age. Then it decreased to 1.63 ± 0.46% at 8.9 months, increased to 2.14 ± 0.62% at 13.5 months, and then decreased again to 0.73 ± 0.17% at 15.3 months. Longitudinal observations were made only for body length and total body weight. Though his cross sectional and longitudinal observations did not always lead to the same conclusions, it seems reasonable to conclude from his data that the body fat component of *N. guentheri* decreases over its lifespan by 1 to 2%. This rather small change in total composition escalates to 5 to 10% of the dry body weight when one assumes a constant 80% body water fraction. Taking the energy value of water-free fat to be 9.5 Kcal/g (Swan, 1974) and assuming the protein composition to be

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approximately constant over the lifespan, we find that a loss in water-free fat of 5 to 10% of the dry body weight corresponds to a net loss in body energy storage of 475 to 950 calories per gram of dry body weight. This is approximately the decrease in body energy over the lifespan shown in the bomb calorimeter data of Table 1. Therefore, unless we have underestimated γ, m or n by a factor of 10 to 100, the small change in the fishes' body fat component appears to be the main source for the variation in body energy content with increasing age shown in this Table. Any body energy decrease with increasing age due to macromolecular crosslinking chemistry appears to be masked by a simultaneous decrease in body fat fraction with increasing age.

SUMMARY

Bomb calorimetry measurements were made on dried whole body samples of *N. guentheri* in an attempt to detect the effect of age induced protein crosslinking. The results show a clear decrease in body energy content with increasing age. However, this decrease was larger than expected and was probably due to a slight decrease in body fat component with increasing age rather than crosslinking alone.

Acknowledgements—The author thanks Mr. T. E. Alberts and Ms. E. L. Tan for their help in carrying out this research. This research was funded by NIH Grant AG 00498.

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Experimental Gerontology, Vol. 17, pp. 145–152, 1982
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MAMMALIAN DESIGN AND RATE OF LIVING

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(*Received* 12 *August* 1981)

Abstract—Terrestrial placental mammals are physiologically and biochemically similar. However, though some of their design and life history characteristics vary in a uniform way with body mass across the mammalian class (*e.g.* basal metabolic rate, body length, liver or adrenal mass, etc.), others follow less uniform "laws" with apparent differences among the various orders (*e.g.* brain mass, life span, etc.). Based on data from 38 species, it is shown that despite the absence of a uniform law describing body mass, m_y, of young at birth, or litter size, n, their product, *i.e.* total body mass of litter at birth, m_λ, does follow such a law: It appears that, like basal metabolic rate, m_λ is proportional to the ¾-power of body mass of the mother; no inter-order differences are apparent. Nevertheless, despite their remarkably similar metabolic apparatus, mammals cannot be functional scale models of each other, *i.e.* the metabolic machinery has to be set at a lower level as body size increases, leading to reduced heart rate, reproductive rate, developmental rate, and "rate of living"—and thus prolonged life span.

INTRODUCTION

AN ELEPHANT does not look like a big mouse; yet, despite many and large external differences, the various species of terrestrial mammals[1] possess a remarkably similar physiological, internal anatomical, and cellular-biochemical organization (Gordon, 1972; Young, 1975). This general mammalian "design" has been modulated in the course of evolution by a myriad of constraints and conditions imposed by the environment. Amidst such evolutionary change (adaptation) though, some principles of mammalian design have remained invariant. In this paper I describe some of these principles, which are at the basis of a discussion of the evolution of mammalian development and life span presented elsewhere (Economos, 1981, 1982).

PHYSICAL FORCES AND LAWS OF PHYSICS AND CHEMISTRY

Like inanimate objects and machines, living organisms are subject to various physical forces and obey the laws of physics and chemistry. However, unlike in inanimate objects which possess only structure and in machines in which structure can exist independently of function, in living organisms structure and function are interwoven and in continuous in-

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[1]Sea mammals, because of their drastically different physical environment, are not considered in this paper.

teraction with each other. Therefore, in organisms, a physical environmental influence is usually exerted on both structure and function.

A strong physical force, constant and uniform in evolutionary time (at least during the evolution of mammals) and to a large extent in ecological space, was gravity. As Galileo pointed out in the early 17th century, because the gravitational load on the legs increases with the size of the mammals, large mammals have proportionally thicker legs. Because small and large mammals are made of similar materials and according to a roughly similar structural design, they obey the principle of mechanical similarity, that is, the distribution of mechanical stress (load divided by surface on which it is applied) is similar in homologous tissues of large and small mammals. Since gravitational load is proportional to body mass and thus to volume or L^3, where L is a characteristic length of the body, while surface is proportional to L^2, for a similar distribution of mechanical stress a disproportional increase of surface, as body size increased, was required. Therefore, gravity has imposed a dissimilarity on mammalian structure (McMahon, 1973, Economos, 1979a).

In the absence of gravity, mammals would be not only geometrically similar but also energetically similar; in other words, the dependence of their basal metabolic rate on body mass would follow a geometrical, surface-law. This follows from the fact that transport of materials and energy to, within, and from the body of an animal (at both the organ and cellular levels) is vectorial through surfaces and its rate is therefore proportional to surface area (Economos, 1979b). However, gravity imposes a metabolic load not commensurate with a surface-law***, leading to a different relationship (Economos, 1979a), the "3/4-power law" (e.g., Kleiber, 1961):

$$E = 70\, m_b^{3/4},$$

where E is basal metabolic rate in kcal/day and m_b is body mass in kg. This equation is valid for all terrestrial placental mammals, with no significant differences among various orders.

Though it is now recognized that it is futile to attempt to define life, it is clear that a basic property of living organisms (which is shared with certain other systems) is their capacity to incorporate matter and energy from the environment and to locally, within themselves, reduce or reverse the rate of entropy production (Morowitz, 1968) by "discarding" some of their entropy into the external environment. *This negative flow of entropy is achieved in the process of cell replication in self-renewing cell lines and in the germ line, or*

***Note added in proof*: My recent research into the origin of biological similarity (Economos, A.C., 1982, *J. theor. Biol.* **94**, No. 25) has led me to a different interpretation (from the one given in this second section) of the role of gravity in the scaling of basal metabolic rate of mammals. First, I have now shown that the argument for obtaining a "surface law" in the absence of gravity is not necessarily true for biological systems (organisms), namely the areas of environment-organism or cell-cell interfaces need not be proportional to external body surface. In fact, it appears that scaling of basal metabolic rate according to a non-surface rule, $m_b^{3/4}$, is dictated by fundamental principles of similarity of mammalian design, various interface surface areas and various processes having been scaled to match this non-geometric scaling. Such a process is related to the metabolic work expended to counteract the deforming effects of gravity: The scaling of basal metabolic rate to obey a 3/4-power rule determines the scaling of metabolic load from gravity according to $m_b^{0.89}$ (Economos, 1979a) and not *vice versa*. An interesting consequence of this scaling has been surmised elsewhere (Economos, A.C., 1981, *J. theor. Biol.* **89**, 211) to be in limiting maximal size of land mammals to about 20,000 kg, which is matched by the weight of the largest known mammal to have existed on Earth, the extinct *Baluchitherium*.

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in the process of protein synthesis and cellular self-repair in both dividing and post-mitotic, differentiated cells. These locally "counter-entropic" processes are driven by the utilization of information: organization that was incorporated in the cellular genetic apparatus during evolution. An analogy can be drawn with a technological system, the refrigerator, in which the engine and cooling system abolish entropy increase locally, *i.e.* in the refrigerated compartment.

Mammals, thus, are not merely heaters. Their cells function as thermodynamic "machines": They use energy from the environment and produce heat at a rate commensurate with their biochemical activities and they actively expel this heat into the environment. Amidst this continual flow of energy they are able to preserve their organization.

Processing of the three different kinds of diet – plant or animal tissues or a combination of the two – required different qualitative modulations of the basic mammalian design. From a thermodynamic point of view, the kind of food may have specific but as yet not well-defined consequences for the organism (Morowitz, 1968; Economos, 1978).

THE STRUGGLE FOR SURVIVAL

A fundamentally different aspect of environmental influence on organisms is related to the problem of availability of food and resulting competition for food as well as the fact that some animals are food to other animals.

Darwin and Wallace realized in the mid-19th century that the struggle for survival is a driving force in the evolution of mammals. Adaptation of a species to a changing environment as well as generation of new species is currently seen to be the result of natural selection which is set by the "drive" for maximal reproductive fitness. Generally, reproductive fitness is analyzed in terms of fitness of the reproductively mature individuals; however, the viability of the newborn young is also important. Elsewhere (Economos, 1982), I evaluate the hypothesis that differences in lifestyle (particularly with regards to birth of the young) and in resulting or co-existing selection pressure from predation or food availability among the various orders of terrestrial mammals, may have influenced various parameters of fecundity (such as gestation period, size of young, and litter size); such adaptations would have operated within constraints inposed by upper limits to metabolic energy production (which is proposed below to apply, for instance, to the total biomass produced per pregnancy).

RATE OF LIVING

Mammals, despite their remarkably similar metabolic apparatus, cannot be functional scale models of each other, that is, the "metabolic machinery" has to be set at a lower level as body size increases. This level corresponds to the specific metabolic rate of the animal and reflects the level of average cellular metabolic activity; it is proportional to $m_b^{-1/4}$ (which is obtained from $m_b^{3/4}/m_b^{1.0}$). The negative exponent indicates that the level of cellular activity is lower the larger the mammal; this is a consequence of design constraints for energy and material transport in the body and not a consequence of decreasing heat loss in larger mammals. Such reduction of specific metabolism in larger mammals has a number of interrelated implications, consistent with the concept of "rate of living" (Pearl, 1928):

(1) Larger mammals are more sluggish, have a lower level of free-running activity and a reduced heart rate;
(2) Their reproductive rate (fecundity) is similarly reduced;

(3) Their rates of prenatal and postnatal development are also reduced; and
(4) Their life span is prolonged.

These implications are discussed elsewhere (Economos, 1982). Below I analyze an implication for size and a metabolic-constraint-upper-limit imposed on the total biomass of newborn young per pregnancy in relation to body size of the mother.

A METABOLIC CONSTRAINT ON MAMMALIAN REPRODUCTION

Because average muscle power of a mammal is supported by its basal metabolic rate, muscle power as well as other forms of energy output of the organism are scaled as basal metabolic rate (Young, 1975). An example of this general principle of biological similarity of mammals is the fact that the total daily milk calories produced by lactating rats and cows (small and large mammals) are the same proportion of their basal metabolic rate (Kleiber, 1965). Reproduction too is a form of energy transformation by the maternal organism. The total biomass of newborn young per pregnancy is equivalent to a number of calories "exported" to the environment by the body of the mother. Therefore, the biomass of a litter at birth may be expected to be the same proportion of the maternal basal metabolic rate in small and large mammals, *i.e.*, proportional to $m_b^{3/4}$. Alternatively, the maximal total basal metabolic rate of embryos at the end of gestation that can be supported by the metabolism of the mother will be proportional to the mother's own basal metabolic rate, or to $m_b^{0.75}$ (similarity). Because each embryo's metabolic rate is proportional to the 1.0-power of its body mass (data, *e.g.*, for sheep are quoted by Brody, 1945), it follows that the total metabolic rate of the embryos is proportional to their total biomass, and this to the mother's basal metabolic rate.

From data on average values of m_y (body mass of young) and n (average size of litter) for 38 species from the four main mammalian orders, rodents, carnivores, ungulates, and primates (See Table 1), values of litter biomass $m_\lambda = n \cdot m_y$ were calculated; these values are plotted in Fig. 1 *vs.* corresponding body mass m_b of mothers. Linear regression of log m_λ *vs.* log m_b gave the statistically highly significant equation ($r = 0.987$)

$$m_\lambda = 0.093\ m_b^{0.774},$$

where m_λ and m_b are in kg. There were no consistent differences among orders.

The exponent of m_b in the above equation is very close to 3/4, that is m_λ obeys the same allometric equation as basal metabolic rate. This trend is statistically highly significant, despite rather large variations in n and m_y.

LITTER SIZE *VS.* BODY SIZE: A MULTIVARIABLE EQUATION

It has been generally assumed that small mammals have larger litter sizes and produce more litters per year than large mammals, to maximize reproductive fitness in the presence of increasing predation with decreasing size. However, this is an incomplete view. For instance, both primates and ungulates give birth, as a rule, to a single young irrespective of the mature body size of the species. Apparently, there are other variables in the reproductive fitness equation which offset the presumed increased predation pressure as size decreases.

Data on average litter size in rodents and carnivores were plotted *vs.* average maternal body size in Fig. 2. There is a weak general trend for litter size to decrease slowly with

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TABLE 1. AVERAGE BODY MASS OF LITTER AT BIRTH (m_λ) *vs.* AVERAGE BODY MASS (m_b) OF THE NONPREGNANT FEMALE IN PLACENTAL MAMMALS

Species name	m_b(kg)	m_λ(kg)	Species name	m_b(kg)	m_λ(kg)
Rodents and lagomorphs			*Carnivores*		
Flying squirrel	.1	.01	Arctic fox	5	.5
Pocket gopher	.22	0.24	Giant panda	110	3
Beaver	16	1.14	Ring-tailed "cat"	.95	.098
Spring hare	4	.25	Raccoon	5.2	.284
Harvest mouse	.014	.004	Striped skunk	1.4	.117
Sagebrush vole	.018	.008	Lion	200	3.9
Mole rate	.153	.015	Mountain lion	60	1.4
Spiny mouse	.063	.0165	*Primates*		
Jumping mouse	.025	.004	Tree shrew	.73	.022
Ungulates			Lemur	1.4	.078
Asiatic elephant	5,000	90	Galago	.315	.041
Indian rhinoceros	3,500	50	Tarsier	.10	.0225
White rhinoceros	2,750	50	Douroucoulis	.73	.10
Hippopotamus	3,500	33	Ateles	5	.5
Pygmy hippopotamus	187	4	Macaque	5	.45
Barking deer	15.3	.6	Orangutan	40	1.5
Wapiti	250	16	Chimpanzee	40	1.9
Mule deer	81	1.7	Gorilla	85	2.1
Okapi	210	16	Woman	65	3.25
Pronghorn antelope	44	2.1			
Musk ox	350	7			

body size, roughly as $m_b^{-0.1}$. However, values for any given body size are widely scattered. Furthermore, there are distinct order-specific differences: A given litter size corresponds to a maternal body mass about 20 times larger in carnivores than rodents. Surprisingly, in both orders litter size increases as adult body size decreases only down to a certain body mass (about 10 kg in carnivores, 0.5 kg in rodents); below this body size, there is a slight tendency for litter size to *decrease*. These trends could be linked to selection pressures from predation in rodents and availability of food for the young in carnivores.

Such an analysis is however inadequate, because it neglects the constraints to which litter size is subject and the consequences it may have for viability of the newborn young. Because there is an upper limit to total litter biomass, $m_\lambda = n \cdot m_y$, at each maternal size m_b, *i.e.*, $m_\lambda \propto m_b^{3/4}$ (see previous section), an increase effected on litter size n by predation pressure would decrease proportionally the size m_y of the newborn young, which is in general detrimental to young viability, thus compromising the improvement in reproductive fitness from increased litter size.

CONCLUSION

Because metabolic-thermodynamic processes underlie organismic aging and set broad constraints on its pace (Economos, 1981), there is justification for an approach to aging using principles of classical and nonequilibrium thermodynamics, modified and adapted to the study of aging. Such an approach should aim *not* at reformulating existing concepts using thermodynamic terminology, which would be a sterile pastime and unconvincing to biologists, but at defining fundamental constraints imposed by the laws of thermodynamics and analyzing life's strategy in overcoming or ameliorating their effects as well as

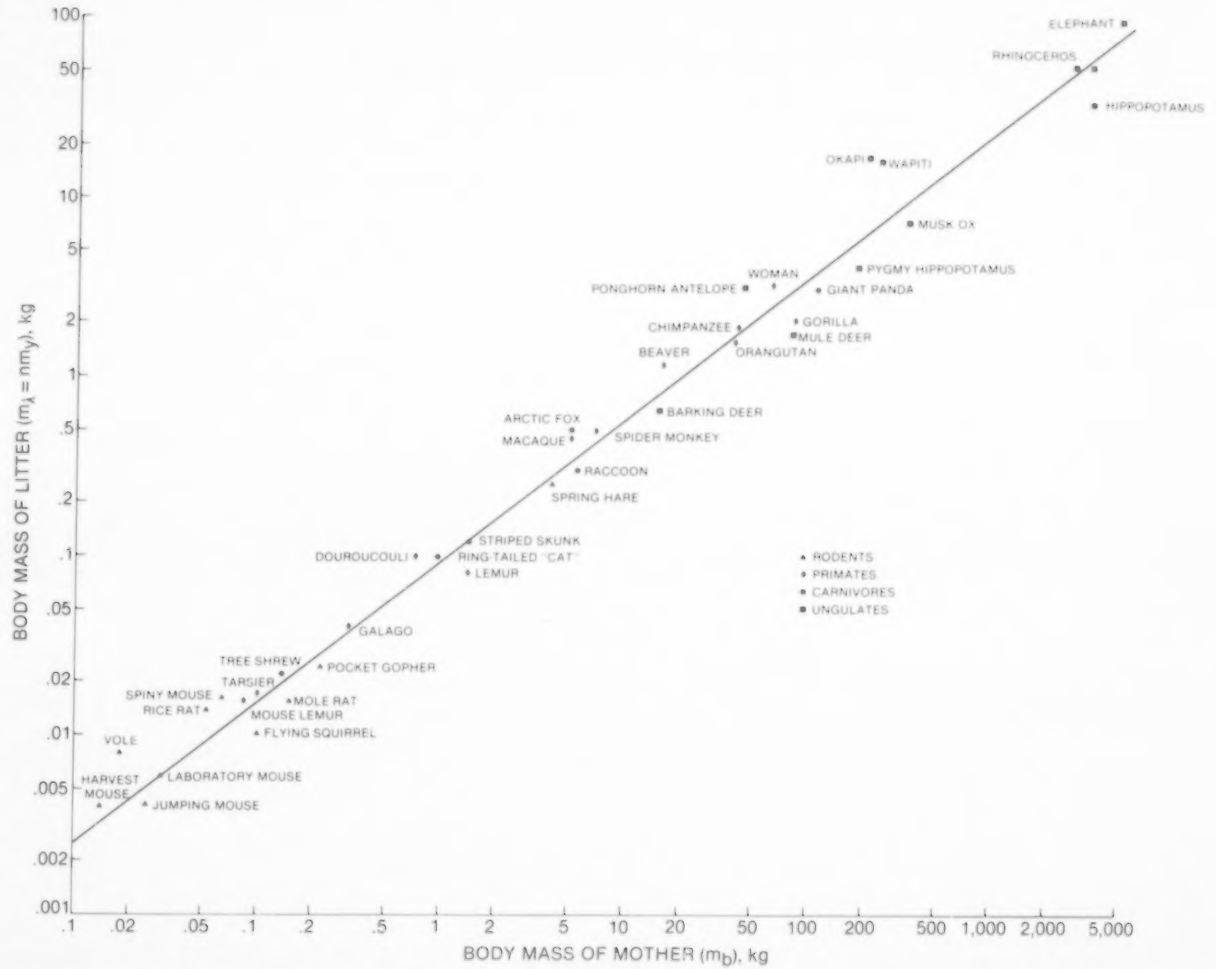

FIG. 1. Correlation of average body mass m, of litter with average body mas m_b of the mature, nonpregnant female in 38 placental land mammals, with representatives from the four main orders, rodents, carnivores, ungulates, and primates. The line was obtained by least square linear regression of log m_λ on log m_b; $r = 0.987$; equation: $m_\lambda = 0.093\ m_b^{0.774}$.

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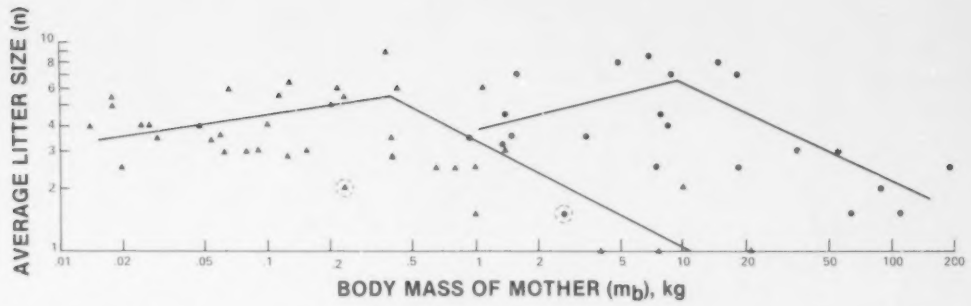

FIG. 2. Dependence of average litter size n on average mature body mass of the nonpregnant female for rodents (left) and carnivores (right); a complex pattern is apparent (see text).

studying consequences of these processes for the stability of cells and organisms. For instance it can be maintained (Economos, 1981)[2] that the rate of animal aging is mainly determined by the overall balance of three basic processes in the organism:

(1) The level of cellular metabolism with which the rate of entropy production in the organism is necessarily proportional;
(2) The degree of effectiveness of protective mechanisms (*e.g.* antioxidant enzyme levels) which reduce metabolic damage to the tissues; and
(3) The capacity of cellular repair mechanisms which repair metabolic damage inflicted on cytoplasmic and genomic constituents.

A fourth, supracellular, mechanism may be involved in determining the aging rate in mammals. Because those cells of the body which continue to divide in the adult organism appear to undergo minimal or no age changes as seen electron microscopically (Miquel *et al.*, 1979), by contrast with fixed post-mitotic cells which show marked senescent deterioration, the presence of dividing somatic cells in mammals as compared *e.g.* with insects which possess only fixed post-mitotic somatic cells, may account partly for the dramatic difference in life spans between mammals and insects. Indeed, the various somatic stem cell lines can be considered as producing "disposable bags" that are continuously discarded in the environment once they have performed their programmed function and have been "filled with entropy" in the process, thus freeing the organism from a considerable part of the otherwise obligatory accumulation of metabolically-linked entropy.

From this analysis it appears that classical theories of aging, such as the "rate of living" and "wear and tear" theories, as well as more modern theories which implicitly or explicitly equate the rate of aging with specific metabolic rate, need reevaluation as to their applicability in mammals (Economos, 1981). Though insect studies clearly support such theories, mammals, unlike insects, possess in addition to fixed post-mitotic cells, various kinds of dividing somatic cells and an intricate system of homeostatic control mechanisms

[2]Note that somewhat similar concepts but in different or less explicit forms have been expressed independently and in various contexts by a number of workers (e.g., Medvedev, 1975; Cutler, 1976; Strehler, 1977; Sacher, 1978, Miquel *et al.*, 1979).

at the organ level, which complicate the testing of these theories as well as interpretation of the results when such testing is attempted. For instance, though an increase in the level of activity causes a decrease in insect life span (*e.g.* Sohal, 1978), this may not be true in mammals, at least for moderate levels of exercise in young adult or middle-aged individuals.

Acknowledgements—I would like to thank various colleagues, in particular Drs. J. Miquel and C.T. Reichwein, for useful discussions on the subject of this and the companion paper, "Evolution of Developmental Rate in Mammals."

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Experimental Gerontology, Vol. 7, pp. 153–158, 1982
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EVOLUTION OF DEVELOPMENTAL RATE IN MAMMALS

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(*Received* 12 *August* 1981)

Abstract—The allometric relationship between gestation period and body mass in terrestrial placental mammals was analyzed using data from 147 species. Important differences were found among the various orders. These differences can be explained if it were assumed that the order-specific lifestyle and environment in which the young are born has caused different selection pressures on the newborn young in the various orders and thus influenced the number of young per litter and duration of gestation. As proposed elsewhere, such evolutionary effects on embryonic development may have indirectly influenced the evolution of mammalian life span.

INTRODUCTION

AN ELEPHANT mother takes 22 months to produce a single baby elephant while a mouse mother can produce as many as 10 baby mice in less than one month; yet, this is not simply because a baby elephant is so much bigger than a baby mouse, for it would take only a few more cell divisions to increase the size of a baby mouse to that of a baby elephant. It can be deduced that since in general the allometric relationships for rates of metabolic processes are governed by a $m_b^{-1/4}$-law (m_b is body mass), as *e.g.*, specific metabolic rate and heart rate (Economos, 1982), and since the period of gestation is the reciprocal of the rate of a metabolic-related process, this period would be proportional to $m_b^{1/4}$. Though such a relationship between body size and gestation period may be roughly true for mammals, an analysis of data from a large group of mammalian species indicated that there are significant and consistent differences among the various mammalian orders. In this paper I propose a hypothesis about the evolutionary basis of these differences and explore some of the implications; an important implication for the evolution of mammalian life span is analyzed elsewhere (Economos, 1981).

INTER-ORDER DIFFERENCES IN GESTATION PERIOD

Data on gestation period, t_{gp}, of 147 mammalian species from the four main orders, rodents, carnivores, ungulates, and primates, were obtained from Walker (1975) and Napier and Napier (1967) and were cross-checked, whenever possible, with data from various other sources in which a single species or a small group of species were described;

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agreement was generally good. These sources contain also general information about lifestyle characteristics and descriptions of the newborn young's stage of development.[1]

Figure 1 shows the data on gestation period t_{gp} plotted *vs.* body mass, m_b. Linear regression equations for $\log(t_{gp})$ on $\log(m_b)$ for the various orders separately are summarized in Table 1. Inspection of the data for primates showed that there were consistent differences between three families of lower primates (*Tupaidae*, *Lemuridae*, and *Indridae*) and the remainder ("higher" primates, including *Anthropoidea* and two Prosimii-families, *Lorisidae* and *Tarsiidae*) which were therefore considered separately.

The slope of the overall regression line, 0.229, is close to 0.25, which was derived above from simple metabolic considerations. Hoever, the slopes of the regression lines for the various orders (except lower primates) are smaller, indicating that within each order gestation period increases with body mass somewhat slower than predicted by that simple metabolic argument.

It has been suggested (Hugget and Widdas, 1951) that the cube root of neonatal weight, m_y, is proportional to gestation period. Based on more extensive data, Sacher and Staffeldt (1974) proposed that the plot of $m_y^{1/3}$ *vs.* t_{gp} is not linear but has a slight upward curvature. In agreement with this conclusion is the relationship $m_y^{1/3} \propto t_{gp}^{1.3}$ which can be obtained from the relations $t_{gp} \propto m_b^{0.23}$ (Table 1) and $m_\lambda = nm_y$, $m_\lambda \propto m_b^{0.77}$ and $n \propto m_b^{-0.1}$ (Economos, 1982), where n is number of young per litter and m_λ total biomass of litter.

The present analysis of gestation periods was initiated in an attempt to explain inter-order differences in the life span-body mass relationship. These differences were not matched by inter-order differences in mature brain size (Economos, 1980b), not are they matched by the differences in gestation period only. For instance, though the lines on the gestation period plot for higher primates and ungulates virtually coincide, higher primates have superior life spans compared with ungulates on a body weight basis, leading to distinct lines on the life span plot.

Kihlstrom (1972) recognized inter-order differences in gestation period and attempted to correlate these differences with the type of placenta, but his analysis was criticized by Sacher and Staffeldt (1974) because of the relatively restricted data base. The latter authors found that gestation time correlated better with neonatal brain weight than with neonatal body weight and suggested that the brain controls embryonic growth. Sacher

TABLE 1. LINEAR REGRESSION ANALYSIS OF LOG GESTATION PERIOD (DAYS) ON LOG BODY MASS (KG) OF MOTHER

Ungulates	Primates	Rodents	Carnivores	All orders
All:	Higher:	All:	$t_{gp} = 50\ m_b^{0.136}$	$t_{gp} = 70\ m_b^{0.229}$
$t_{gp} = 106\ m_b^{0.185}$	$t_{gp} = 157\ m_b^{0.089}$	$t_{gp} = 58\ m_b^{0.234}$	$r = 0.797$	$r = 0.761$
$r = 0.824$	$r = 0.745$	$r = 0.816$		
Without suidae and hippopotamus:	Lower:	Smaller than 1.5 kg:		
$t_{gp} = 106\ m_b^{0.173}$	$t_{gp} = 99\ m_b^{0.318}$	$t_{gp} = 50\ m_b^{0.179}$		
$r = 0.715$	$r = 0.867$	$r = 0.578$		

[1]It should be emphasized that only general trends in terrestrial placental mammals are considered here. Because of the many degrees of freedom and large variation in environmental conditions (including predation pressure and food availability) some species deviate from these trends. For instance, some families, such as *Mustelidae* and *Ursidae*, have delayed implantation, which artificially prolongs the gestation period; such species were not considered in the present analysis.

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(1959) had previously proposed that the brain controlled life span, based on a similar statistical analysis, but I have shown that this conjecture was not valid (Economos, 1980a; 1980b). Sacher and Staffeldt did not show if the brain's correlation with gestation period is unique, *e.g.*, how does neonatal liver or adrenal weight correlate with gestation time? Further, because primates and ungulates give birth usually to a single young, whereas in rodents and carnivores larger litter sizes are the rule, neonatal weights of one young only are not comparable. Finally, if indeed brain size were a (the) determinant of the rate of embryonic growth, what then determines brain size?

GESTATION PERIOD AND SPECIES LIFESTYLE: AN HYPOTHESIS

There are distinct differences in the stage of development of newborn young in the various mammalian orders (Walker, 1975). The single (as a rule) ungulate young is able to stand, obtain milk from its standing mother, and follow the herd within one to two hours after birth. The single primate young, however, is unable to walk and "care for itself", but its striking characteristic is its disproportionately large head. Finally, the multiple young of carnivores and rodents are generally blind and helpless at birth (see Footnote 1).

Because ungulate young are usually born in the open field and are thus exposed to predators, there is a strong selective pressure for the young to be able to run as soon as possible after birth. In addition, the young have to be able to stand to obtain milk from their mothers and follow the group soon after birth in their constant wandering around for food and avoidance of predators. A selection of ungulates for newborn young that are as mature as possible may therefore have taken place, because immature young, being unable to follow the adults, were preyed upon by the carnivores and eliminated, or were not able to get enough milk from their mothers and starved. (The latter type of selection pressure is most pronounced in the giraffe because of the well-known vulnerability of the adult animal in the lying position.) One consequence of such selection was to reduce the number of young to the smallest possible number, usually one or rarely two. In addition, the gestation period was significantly increased compared with that of equally sized carnivores by almost 2.5 times according to Fig. 1. The achieved t_{gp}s probably represent an upper limit because a longer gestation period accompanied by an equal rate of embryonic growth would lead to a young too large to be supported by the metabolism of the mother (upper limit established elsewhere, see Economos, 1982).

Being born in nests or burrows, the young of carnivores and rodents are protected. Therefore, there is not a strong selective pressure for them to be born as mature as possible—they are born blind and helpless (with a few exceptions). However, later in life, rodents are heavily preyed upon and there is a scarcity of food for carnivores from birth on, so that there is selective pressure to increase the number of young per litter in these animals and decrease the production period ($\downarrow t_{gp}$); this pressure is generally stronger the smaller the adult body mass of a given species. This is only a trend, however; the detailed dependence of number of young per litter on m_b is rather complex (Economos, 1982). In short, the average litter size of rodents is largest for species with m_b near 0.5 kg and *decreases* slowly as m_b decreases but rapidly for increasing m_b. This correlates with the tendency of large rodents (*e.g.*, *hystricomorpha*) to have t_{gp}s considerably longer than predicted from the trend in small rodents. Selection of the large rodents for fewer young per litter but longer t_{gp} and thus better developed young at birth, is similar to, but less pronounced than in ungulates, and can be accounted for by the change in predation pressure

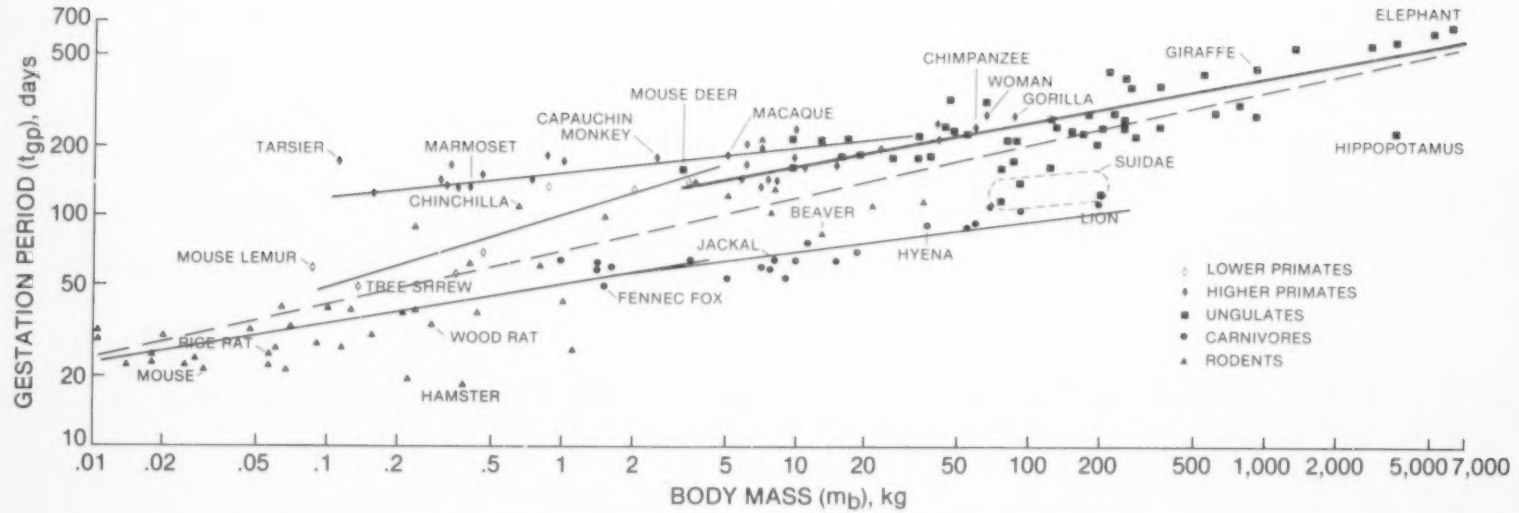

FIG. 1. Correlation of gestation period with mature body mass of the nonpregnant female for 147 terrestial placental mammals; regression lines are drawn for the various orders separately (see Table 1).

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accompanying the increase in size. By contrast, although carnivores have a similar pattern of dependence of number of young per litter on body mass as rodents (the peak occuring however near 10 kg), they show no corresponding increase of young size at birth or disproportionate increase of t_{gp} above a body mass of 10 kg; There is no strong selection pressure for better developed newborn young. Indeed, whereas more mature young of large rodents can obtain food and avoid predators more effectively, better developed young of carnivores would still depend on the parents for food. Thus, although the dependence of t_{gp} on m_b in small rodents and carnivores is similar to that of ungulates (indicated by the parallel regression lines in Fig. 1), carnivores and small rodents were apparently selected for a shorter t_{gp}, larger rodents for an intermediate t_{gp}.

Of all ungulates, suids appear to follow the "rodent-carnivore law"—multiple births and shorter t_{gp}s (see Fig. 1)—may be (but not exclusively) due to their nocturnal lifestyle, the birth of the young in protected places and the heavy predation. The "rationale" for the striking deviation from the ungulate line represented by the hippopotamus may be as follows. The young hippopotamus, which is considerably smaller than the young rhinoceros for instance (despite the comparable weight of the mothers), is born in the water and thus is protected from predators, it does not have to stand or to run on land (which it is unable to do) and can readily obtain milk from its mother by periodically diving in and out of the water (Walker, 1975). These aspects of the hippopotamus' environment and lifestyle may have led to its selection for a smaller young and a shorter t_{gp} than other ungulates of comparable size (in fact, absence of selection in the opposite direction).

Finally, the young of many primate species are also born in protected places, usually in nests on trees. They are initially carried around by their parents, which is possible in primates because the young can cling onto their parents' bodies. Therefore, there is not a selective disadvantage against their being born with immature musculature and unable to run. Because the adults carry the young and can protect them from predators by climbing into trees, the chances of the young to survive to sexual maturation are good, and there is not a selective advantage of multiple births. Under these conditions, an increased brain capacity would apparently increase the chances of living to sexual maturation and would be selected accordingly. Again, it is mainly the primates' lifestyle that has favored single births and increased prenatal growth of the brain--a prerequisite to a larger adult brain—and thus selected a gestation period as long as possible. In this way, t_{gp} in primates reached similar proportions as in ungulates, that is it reached the upper limit of support by the metabolism of the mother. However, the group of lower primates, *Prosimii*, appears to cross-over from the rodent-carnivore line to the higher primate-ungulate line. At the beginning of the *Prosimii* line we find the tree shrews, the family *Tupaidae*, which have a gestation period very close to that of rodents of similar body mass. Because insectivores have gestation periods similar to rodents, this finding is in agreement with the characterization of *Tupaidae* as a "link between insectivores and primates" (Buettner-Janusch, 1971).

CONCLUSION

It appears, therefore, that differential selection pressures from differences in lifestyle among primates, rodents, carnivores, and ungulates, have profoundly influenced their gestation period and rate of embryonic development. *Homo sapiens*, though not different from other higher primates in this respect, has been additionally selected for language development that led to further evolution of the brain, according to Monod (1971).

Because natural selection may in this way have affected the rates of embryonic and postnatal growth of mammals, it may indirectly have influenced their lifespan (Economos, 1978; 1981).

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Experimental Gerontology, Vol. 17, pp. 159–162, 1982
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ALTERED ACTIVATION OF RAT HEPATIC CYCLIC AMP-DEPENDENT PROTEIN KINASES WITH INCREASING AGE

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(*Received* 23 *July* 1981)

INTRODUCTION

ONE GENERAL feature of all aging populations is their progressively modified ability to adapt to changes, *i.e.*: stresses in the surrounding environment. On the physiological level, this is seen as decreases in basal metabolic rate, maximal breathing capacity, glomerular filtration rate, etc. (Shock, 1962). On the biochemical level, this decreasing ability to respond to stress includes an altered ability to initiate adaptive fluctuations in the activities of a variety of enzymes (Adelman, 1975). That is, with increasing age, some enzymes are altered in the time-course of response, some in magnitude of response, and others in both time-course and magnitude of response (Adelman, 1975).

Increased enzyme activity may be a function of either the activation of pre-existing enzyme molecules or the synthesis of new enzymes. Both of these processes have been linked to the activation of the cyclic AMP-dependent protein kinases (Langan, 1973; Dokas and Kleinsmith, 1971). One enzyme that has been shown to be induced in response to the activation of the cyclic AMP-dependent protein kinases is ornithine decarboxylase (ODC) (Byus and Russell, 1975). Moreover, a positive correlation has been demonstrated between the degree of activation of the cyclic AMP-dependent protein kinases and the magnitude of the ornithine decarboxylase response (Byus *et al.*, 1976; Russell and Byus, 1976).

With advancing age, ODC activity in rat liver, in response to injected growth hormone, exhibits both an increased lag time and decreased maximal response (Russell *et al.*, 1970). To explain this result, we postulated that the altered response of ODC with increasing age is due to a change in the cyclic AMP-dependent protein kinases.

In this study, we report decreased activation of rat liver cyclic AMP-dependent protein

This study was supported in part by awards from: [1]University of Alabama Student Government Association Venture Fund, and [2]Research Grants Committee, Award #1044.

kinases with increasing age. We propose that this decreased activation is a primary mechanism in the biochemistry of aging.

MATERIALS AND METHODS

Male, Long-Evans rats of 28, 68, and 300 days of age were purchased from Zivic-Miller, Inc. The animals were maintained for one week at a constant 27°C, 10 h dark/14 h light schedule, with food and water *ad libitum*. The animals were killed by cervical dislocation, and their livers were removed and frozen between blocks of dry ice within 15 sec of death. Tissues were stored at −70°C until assayed.

Frozen tissues were homogenized in 5 vol of 0.05 M Tris-Cl buffer (pH 7.5, containing 0.01 M $MgCl_2$, 0.01M NaF and 0.003 M EGTA) and centrifuged at 9,000 × g for 2 min at 4°C. The supernatant was diluted with buffer to yield a suspension of 50 ug protein per 25 ul. Diluted supernatants (25 μl) were assayed in the same buffer for protein kinase activity in a total volume of 75 μl containing (γ-^{32}P) − ATP (0.01 uCi) and 100 ug of casein in the presence and absence of 5 uM cyclic AMP. The samples were incubated for 5 min at 30°C, and the reaction was stopped by spotting a 60 ul aliquot onto a Whatman 3 MM paper filter. Filters were washed three times (20 min each) in cold 10% TCA, once in 95% ETOH and counted in toluene scintillation fluid (Mao and Guidotti 1974; Corbin, *et al.*, 1975).

The activity ratio is expressed as the amount of ^{32}P − ATP incorporated into casein in the absence of cyclic AMP divided by the amount of ^{32}P − ATP incorporated in the presence of cyclic AMP (− cyclic AMP/ + cyclic AMP).

Protein concentration was determined by the Bradford dye-binding method (Bradford, 1978), using bovine serum albumin as the standard. Casein and cyclic AMP were purchased from Sigma; (γ ^{32}P) − ATP (2300 Ci/m mole) was obtained from New England Nuclear.

Statistical difference was determined by analysis of variance.

RESULTS

The degree of activation (activity ratio) of cyclic AMP-dependent protein kinase represents the relative proportion of the protein kinase present as free catalytic subunit, and is determined by assaying liver cell supernatant kinase activity in the presence and absence of saturating amounts of cAMP (Mao & Guidotti, 1974; Corbin *et al.*, 1975).

The degree of activation or activity ratio, as measured in the livers of normal rats, decreases with increasing age. This is seen as an increasing activity ratio when protein kinase activity is measured in the absence and presence of exogenous cyclic AMP (Table 1).

The activity ratio observed for 35-day-old rats is 0.76, relatively high when compared to a ratio of 0.50 reported by Costa *et al.*, (1975) for rats weighing only 100 g (age probably 25 days). The activity ratio increased to 0.92 for rats 75 days old, and to 0.98 for 300-day-old rats. These values represent a significant change ($P > 0.05$) in the activation of the cyclic AMP-dependent protein kinases with increasing age.

DISCUSSION

This study is of particular interest because it is the first to correlate age and hepatic cyclic AMP-dependent protein kinase activity. Several previous studies have reported changes in the cyclic AMP-dependent protein kinases during embryogenesis and postnatal development (Lee *et al.*, 1976; Bernard and Wasserman, 1977; Shambaugh *et al.*, 1978; Lee and Jungmann, 1975; Haddox *et al.*, 1979). Correlation of their research and our data indicate that changes in the cyclic AMP-dependent protein kinases may be ongoing throughout the lifetime of an individual.

Cyclic AMP plays a role in numerous metabolic processes including processes which directly involve transcriptional activity and the induction of nuclear RNA (Langan, 1973; Dokas and Kleinsmith, 1971). The expression of the effects of cyclic AMP is mediated via the regulation of cyclic AMP-dependent protein kinases (Kuo and Greengard, 1969);

TABLE 1. CHANGES IN HEPATIC CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY RATIO WITH INCREASING AGE

Age in days	Body wt. (g) $\bar{X} \pm$ S.E.	N	Cyclic AMP-dependent protein kinase activity ratio (−cAMP/ + cAMP) ^{32}P cts incorporated into protein/sample −cAMP	+cAMP	$\bar{X} \pm$ S.E.
35	135 ± 3.1	5	3,363	4,417	0.76[a] ± 0.03
75	319 ± 3.9	5	4,854	5,275	0.92 ± 0.06
305	646 ± 34.1	5	5,164	5,280	0.98[a] ± 0.05

[a] $F = 4.33$ between values sharing superscript.

therefore any alteration in these enzymes would produce remarkably diverse effects. These effects would not necessarily be constant from cell type to cell type, nor stimulus to stimulus, since specificity of response depends upon the substrate phosphorylated as well as the type and amount of cyclic AMP-dependent protein kinases. Since the ability to initiate adaptive fluctuations in activities of more than 50 enzymes is altered during aging, and those effects are varied (Adelman, 1975), alterations in the amount or activation potential of cyclic AMP-dependent protein kinases with age could be responsible for the diverse effects observed.

We propose that changes in the amounts, types, or activation potential of the cyclic AMP-dependent protein kinases are an ongoing phenomenon. The decrease in the proportion of free catalytic subunits could be directly responsible for the altered ability to initiate adaptive fluctuations in enzyme activity seen with advancing age, which, in turn, may be directly related to the physical manifestations of aging.

SUMMARY

The activity ratio of hepatic cyclic AMP-dependent protein kinase has been found to change with increasing age in male, Long-Evans rats. In rats 35 days old (135 g), the activity ratio (cyclic AMP-dependent protein kinase activity measured in the absence and presence of exogenous cyclic AMP) was 0.76, it increased to 0.92 in rats 75 days old (315 g) and to 0.98 in rats 305 days old (646 g). This change in the activity ratio seems to correlate with the ability of aging animals to respond to a stimulus, and may be a primary mechanism in the biochemistry of aging.

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Experimental Gerontology, Vol. 17, pp. 163–172, 1982
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LIFE SPAN OF THE WILD AND MUTANT NEMATODE *CAENORHABDITIS ELEGANS*

Effects of Sex, Sterilization, and Temperature

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(*Received* 1 *October* 1981)

INTRODUCTION

THE FREE living nematode *Caenorhabditis elegans* has suitable qualities for aging studies, because it has a simple body structure with the lowest number of somatic cells among multicellular organisms, is easily cultured in the laboratory, and has a short life span. Furthermore, the embryonic and post-embryonic cell lineages of the organism have been extensively worked out (Sulston and Horvitz, 1977; Deppe *et al.*, 1978), and the life cycle has been well studied (Byerly *et al.*, 1975; Hirsh *et al.*, 1976). It is also a favorable organism for genetic analysis, since a large number of chromosomal markers were mapped and mutants are easily obtainable (Brenner, 1974). Thus, *C. elegans* is one of the most promising experimental animals for the study of aging from the genetical and cytological standpoint.

Little is known about the factors that influence the survival of *C. elegans*. The mean life span of worms grown on agar at 20°C with bacteria as the food source was 2 weeks, but in liquid it was dependent on the food concentration (Klass, 1977). If the first stage larvae are deprived of food, they will enter into the dauer stage following the second moult, and there is an increase in the mean life span by several-fold (Klass and Hirsh, 1976). Little, if anything, is known about other factors altering the life span of the nematode. Establishment and characterization of sterile worms is essential for aging study at the individual cell level since self fertilizing hermaphrodites have many eggs which are obstacles to both microscropic observation and the manipulation of individual somatic cells.

In this paper, we studied the life span variations of nematodes, comparing animals of

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different sexes at various temperatures, sterilized wild animals, and temperature sensitive-sterile mutants, to provide the basis for a more detailed genetic and cytological analysis of aging nematodes, and showed that the life span of *C. elegans* was rigidly determined by somatic cells and influenced by temperature effects on the cells at the post-mitotic state.

MATERIALS AND METHODS

Nematode strains and growth

Caenorhabditis elegans, var. Bristol, strain N2, was obtained from Dr. H.F. Epstein, who, in turn, obtained his stock from Dr. S. Brenner. Nematodes were cultured on Petri plates of NGM agar seeded with *Escherichia coli* strain OP50 as described by Brenner (1974). Temperature-sensitive (ts) abnormal embryogenesis mutant HC61 (emb-5) and HC70 (emb-9) were isolated following ethyl methane sulfonate treatment. HC61 is also gonadogenesis defective when individuals are cultured at 25°C before L4 stage and HC70 stops growing when placed at 25°C in any stage between newly hatched juvenile and L3. Developmental and genetic characterization of the mutants were described before (Miwa *et al.*, 1980; Schierenberg *et al.*, 1980).

For survival studies, individual worms were grown on Petri plates (3.5 cm diameter) with NGM agar with a central spot of bacterial lawn and transferred daily to fresh plates using a toothpick. If the worm did not respond to a light touch with a toothpick, the worm was considered dead. Body length was measured with an eyepiece graticule under an Olympus dissecting microscope after the worm was killed by heat treatment.

Synchronous cultures for life span determination were obtained by collecting the first stage larvae that hatched during 2 hours as described before (Hosono *et al.*, 1980).

Fluorodeoxy-uridine treatment

Exposure of worms to 5-fluorodeoxy-uridine (FdUrd) was carried out by the procedure of Hosono (1978) and is summarized as follows. A culture of *E. coli* OP50 was washed with M 9 buffer by centrifugation and suspended at a density of about 2×10^{10} cells/ml in the same buffer containing 5 mM of FdUrd and uridine. A Petri plate with bactopeptone-omitted NGM agar (NP plate) was innoculated with one drop of the bacterial suspension and kept at 37°C overnight before use. After washing on Millipore filters (8.0 μm pore size) with M9 buffer, worms were transferred to FdUrd-containing plates.

RESULTS

Survival of male and hermaphrodite C. elegans

Fig. 1a shows the survival curves of wild-type male and hermaphrodite *C. elegans*. A kinetic analysis of the survival curves present useful proof concerning how the life span is determined in the organisms. Fig. 1b gives the result of a kinetic analysis in which the survivals were plotted semi-logarithmically. The dying segment of the curve is composed of three phases. The first and last phases are influenced by factors not intrinsic to the aging process in itself, such as endotokia (death caused by uterine larval progeny), accidental damage, etc. In the second dying phase, around 50% survival is considered to be most appropriate in kinetically analyzing the aging population because the population decreases almost linearly during this phase. We determined the half-life of the population ($T^{1/2}$) from the linear region of the dying phase and the beginning of dying phase (P) from a cross point of the extrapolated linear line of the dying phase and the 100% survival level as shown in Fig. 1b. These values for both sexes are shown in the first section of Table 1, indicating similar 50% survival times but different P and $T^{1/2}$ values. Some adult hermaphrodites have in their uteri larval progeny that consume parental tissues, and as a result, they almost die during their reproductive period (endotokia). If this population is removed, the beginning of dying phase (P) is slightly retarded but $T^{1/2}$ is the same (Table 1).

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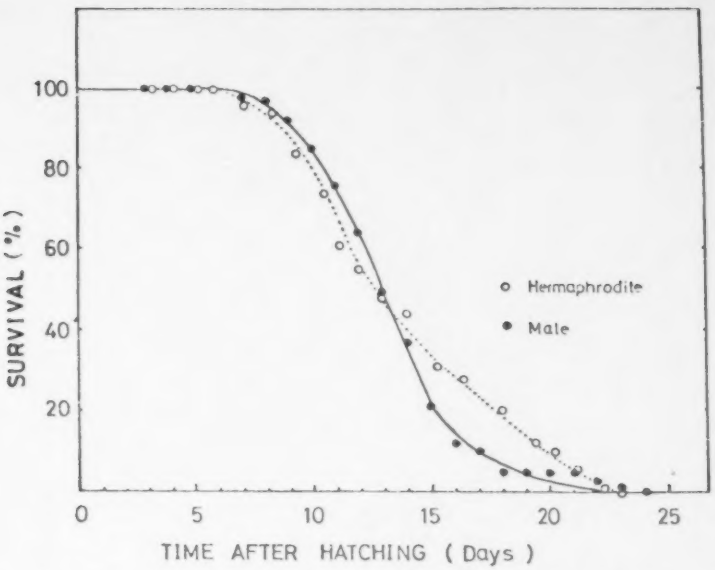

FIG. 1a. Survival curves of hermaphrodite and male *C. elegans* at 20°C. Worms were individually reared on NGM agar plates and transferred to fresh plates daily. Starting male population contained 180 worms and hermaphrodite 200 worms.

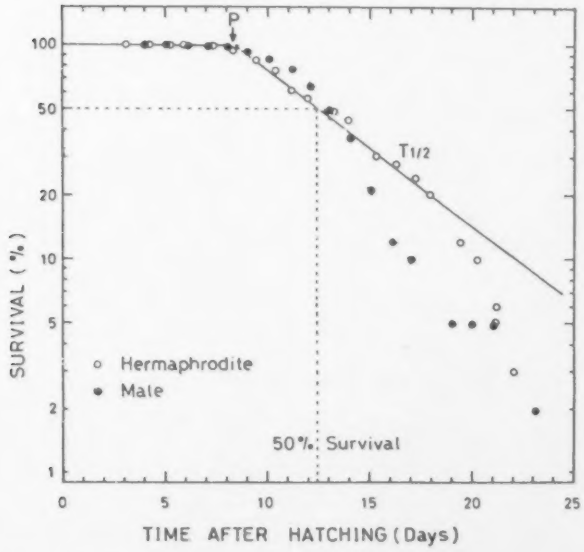

FIG. 1b. Semi-logarithmic plot of hermaphrodite and male survivals from the results in Fig. 1a. The positions of *P* and 50% survival time are shown in the case of hermaphrodite.

TABLE 1. THREE PARAMETERS IN SURVIVAL CURVES REFLECTING THE EFFECTS OF SEXES AND STERILIZATION

Condition	P* (days)	$T^{1/2}$** (days)	50% survival (days)	See also
20°C				
male				
hermaphrodite	11.0	1.8	12.8	Fig. 1
total worms	8.5	4.2	12.5	
worms without endotokia	9.3	4.2	13.5	
25°C				
wild N2	5.0	2.8	7.8	Fig. 3
mutant HC61	6.2	3.3	9.5	
25°C				
untreated control	7.0	5.5	12.7	Fig. 4
5 mM FdUrd treated	9.3	3.3	12.7	

*P = dying phase.
**$T^{1/2}$ = half-life.

Survival of ts mutants and FdUrd-treated C. elegans

Sterile hermaphrodite survivors were examined through the use of temperature sensitive (ts) mutants or DNA synthesis inhibitor-treated worms. A mutant, HC61, grows normally at 16°C, but is defective in gonadogenesis at 25°C. At 25°C the mutant grew at a normal rate during the early development but was slightly smaller than the wild-type at adult phase (Fig. 2). Compared to the wild-type hermaphrodite, the mutant population began to decrease with slightly higher P and $T^{1/2}$ values and had a similar maximum life span (Fig. 3 and Table 1). The survival pattern of another temperature sensitive mutant, HC70, was also approximately the same as of the wild-type (data not shown). As previously reported by Hosono (1978), FdUrd prevented maturation of the reproductive system without influencing the growth of *C. elegans*. The worms were cultured on NP agar as described in Materials and Methods, and the life span of *C. elegans* with or without 5 mM FdUrd was followed. Presence of FdUrd caused slight changes in P and $T^{1/2}$ values giving approximately the same means and maximum life spans (Fig. 4 and Table 1).

Effect of temperature on survival

Fig. 5a shows the effect of temperature on survivors. An increased temperature caused a decrease in both the 50% survival stage and the maximum life span with decreased P and $T^{1/2}$ values (Table 2). If these curves are reconstructed, however, based on the stages of 50% survival, the survival patterns were similar to each other (Fig. 5b).

In order to characterize temperature-sensitive stages, the effects of upward and downward shifts of temperature on survivors were examined at different stages of the life cycle (Fig. 6a and 6b). An increase in life span always involved low temperatures during the dying phase, but low temperatures during the growing phase did not affect the life span. High temperature treatment during the growing or adult phases brought about an

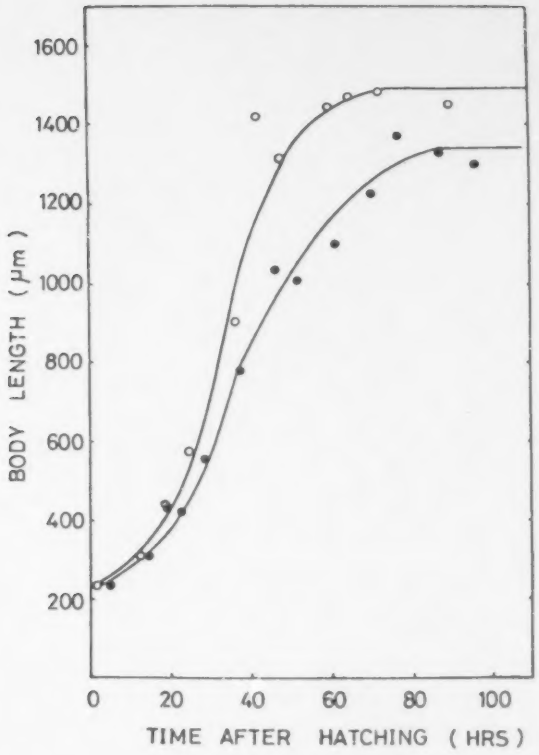

FIG. 2. Growth of wild type (N2) and mutant (HC61) hermaphrodites at 25°C. Worms were grown on NGM agar containing bacterial lawn. At the indicated times, worms were suspended in one drop of M9 buffer on a glass slide, heated slightly, and body lengths were measured with an eyepiece graticule. ○ = N2; • = HC61.

earlier onset of the dying phase (decrease in P value). Temperatures shifting downwards at the adult phase, however, caused a great increase in the half-life of the population (their $T^{1/2}$ values were much higher than the $T^{1/2}$ values of the low temperature control group), resulting in maximum life-spans similar to the control group.

DISCUSSION

Nematodes have been extensively used for the study of development and aging (Epstein and Gershon, 1972; Brenner, 1974; Edgar, 1977), but the use of hermaphrodites is complicated by rapid reproduction (parents become indistinquishable from their offspring within a few days) and by parental death due to endotokia. These problems can be dealt with by frequent transfer of worms being tested, and by using males or sterile hermaphrodites. Although the appearance of males in a natural population is rare, a synchronized male population is collected by using mating plates with a high ratio of male to hermaphrodite and it is also possible to use *him* mutants that produce a high frequency of

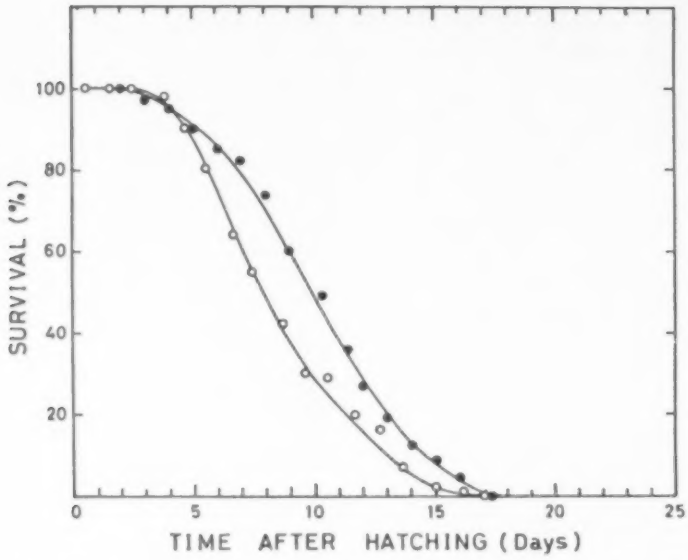

FIG. 3. Survival curves of N2 and HC61 hermaphrodite at 25°C. ○ = N2; • = HC61.

males (Hodgkin *et al.*, 1979). Adult males often died of accidental causes, however, such as crawling up the dish wall, and therefore the recovery of a population that has been observed througout their life spans is very low. By using the difference in sensitivity to FdUrd between gonad and somatic cells, we can obtain sterilized animals without influenc-

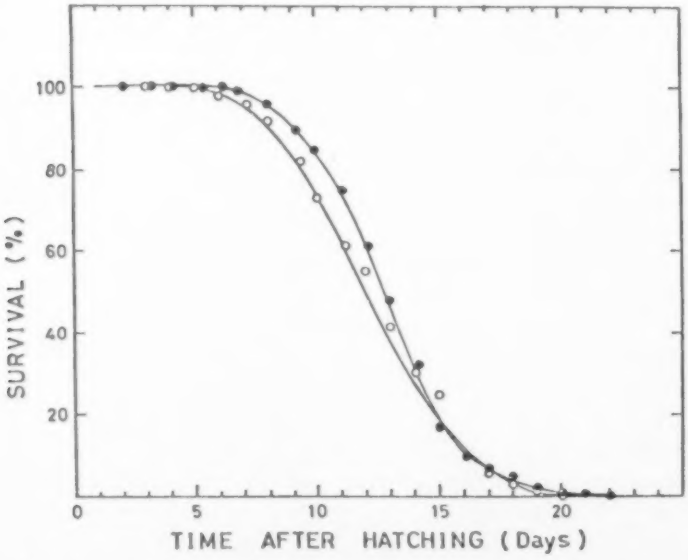

FIG. 4. Effect of FdUrd on survival of N2 hermaphrodite. Procedures were as in Fig. 1, except that NP plates were used. ○ = control; • = 5mM FdUrd and 5mM Urd containing *E. coli*.

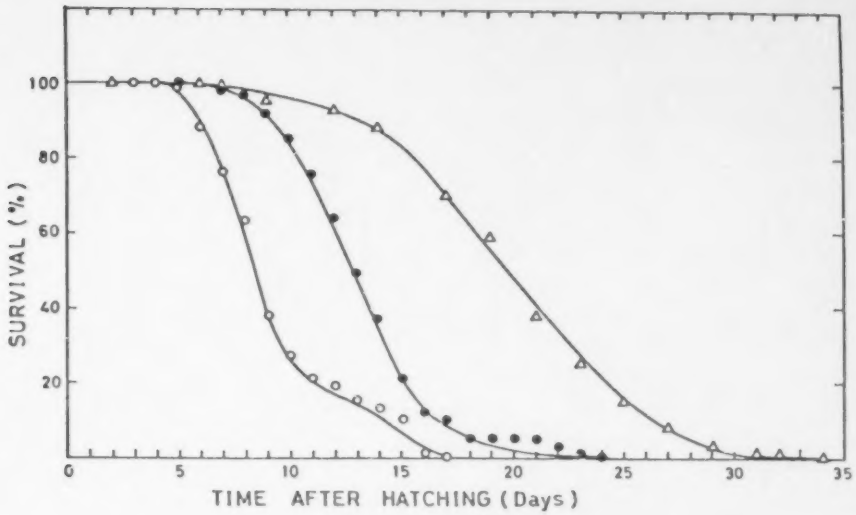

FIG. 5a. Survival curves of N2 hermaphrodite at 16°C(△), 20°C(•) and 25°C(○).

ing growth and life span. The application of this method to the study of aging is limited, however, as the influence of this drug on other living systems is unknown. Many temperature-sensitive developmental mutants have been obtained in *C. elegans*. Although the culture temperature is limited, such mutants are especially useful for aging experiments.

In the present experiments, we showed that male, FdUrd-treated sterile hermaphrodites and temperature sensitive mutants with defective gonadogenesis had approximately the

TABLE 2. TEMPERATURE EFFECTS ON THREE PARAMETERS IN SURVIVAL CURVES

Condition	P* (days)	$T^{1/2}$** (days)	50% survival (days)	See also
no shift				
16°C	16.0	3.7	19.7	Fig. 5
20°C	9.5	3.1	12.6	
25°C	5.5	2.8	8.2	
up-shift from 16°C to 25°C at each phase				Fig. 6a
L4 (2.9 days)	7.2	2.0	9.2	
Adult (5.0 days)	8.3	1.4	9.7	
Dying (16.7 days)	17.1	1.6	18.0	
down-shift from 25°C to 16°C at each phase				Fig. 6b
L2 (1.3 days)	8.3	13.7	22.0	
L4 (2.5 days)	4.5	14.0	18.7	
Adult (5.0 days)	6.3	6.7	13.0	

*P = dying phase.
**$T^{1/2}$ = half-life.

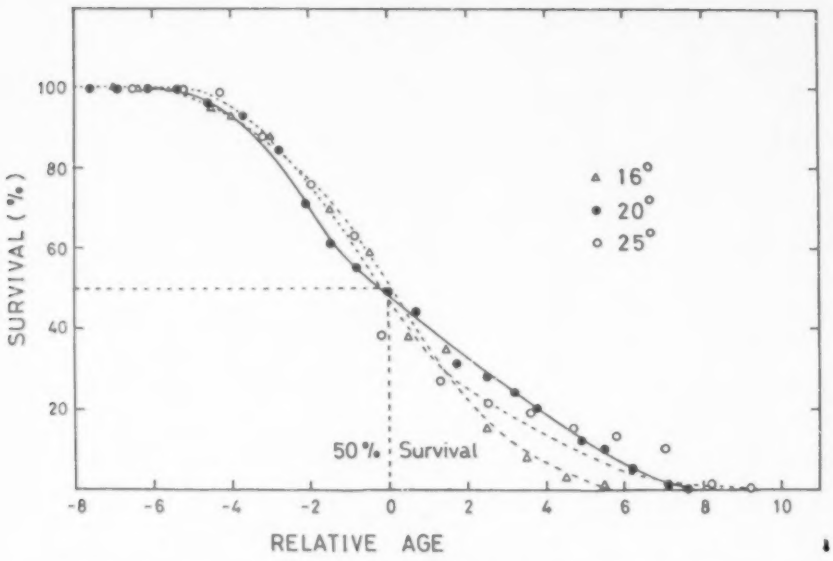

FIG. 5b. Survival curves plotted against relative age in Fig. 5a. Ages between hatching and 50% survival stage were divided into ten equal parts. Then, the survival curves were reconstructed using this relative age and expressed on the basis of 50% survival stage.

same 50% survival rates and maximum life spans as compared to their hermaphroditic counterparts, indicating that the life span of the nematode *C. elegans* is mainly determined by somatic cells.

On the other hand, low temperature treatment markedly extended the 50% survival and maximum life-spans as Klass *et al.* reported (1977). The extension of life spans was accompanied by an increase in P value (beginning of dying phase) and $T^{1/2}$ value (half-life of the population in the dying phase). Finding that the patterns of the survival curves are similar to each other when compared on the basis of the 50% survival stage, however, suggests that life span might be a function of rate of metabolism that is determined by the body temperature of the poikilothermic nematode.

The temperature shift experiments indicate that the results of a downward shift are not necessarily the means of high and low temperature control groups, as seen in the much higher $T^{1/2}$ value in the downshift group compared to the low temperature control. High temperature treatment during development caused an earlier start of the dying phase but a downshift of temperature after that brought about a longer half-life of the population in the dying phase, giving the same maximum life span as that of the low temperature control. Thus, temperature shift experiments disclosed marked variations of life span in individual worms in a harsh environment, although mean life span is rigidly determined. Furthermore, these experiments revealed that an increase in life span always involved low temperature after maturation and that low temperature during development did not affect the life span. Thus, we conclude that the life span of *C. elegans* is rigidly determined by somatic cells and influenced markedly by temperature effects on the cells at the post-mitotic state. Therefore, manipulation of somatic cell activity at the post-mitotic state us-

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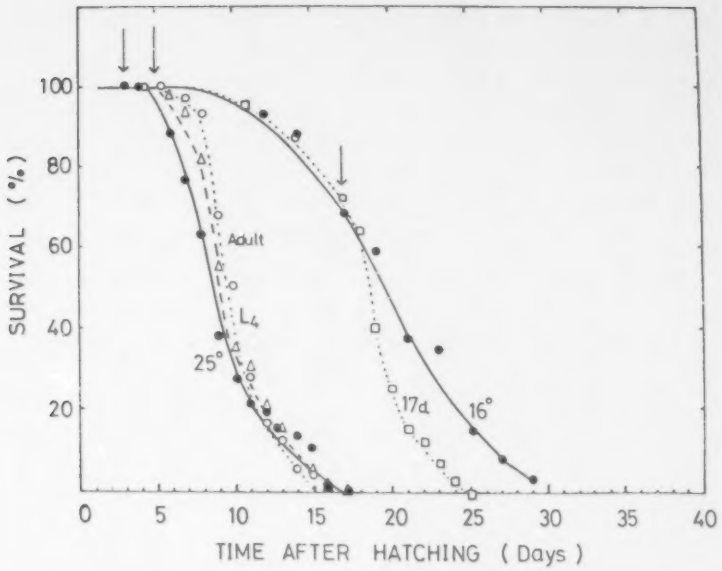

FIG. 6a. Temperature up-shift effect on survival. N2 hermaphrodites were grown on NGM agar and transferred from 16°C to 25°C incubator at L4 (△), adult (○) or dying phase (□).

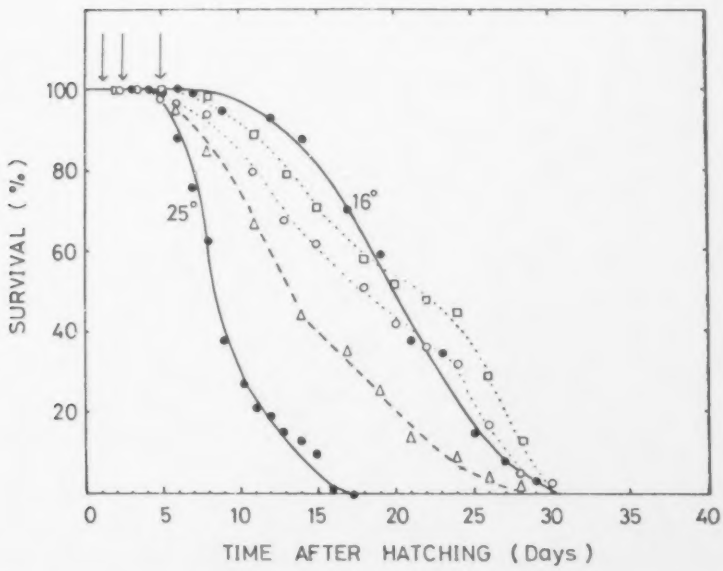

FIG. 6b. Temperature down-shift effect on survival. The worms were transferred from 25°C to 16°C at L2 (□), L4 (○) or adult phase (△). The exact times of temperature shift are shown in Table 2.

ing temperature sensitive sterile mutants might be a promising approach to examining the role of post-mitotic cells in the aging of an organism. In a previous paper, we examined several aging parameters fractionating an age-homogenous populaton of nematodes with high individual variations in life span. We are currently developing a system to manipulate individual somatic cells of ts mutant sterile nematodes examining their age parameters and life spans.

SUMMARY

The survival of *Caenorhabditis elegans* was studied comparing animals of different sexes, sterilized animals, and animals grown at different temperatures as a prelude to more detailed cytological and genetic analysis of aged nematodes. Temperature-sensitive sterile mutants, animals sterilized by 5-fluorodeoxyuridine treatment, and wild-type males showed little difference in life span from that of wild-type hermaphrodites, although slight changes in P (time of beginning of the dying phase) or $T^{1/2}$ (half-life of the population in the early dying phase) values were observed. At higher temperatures, P and $T^{1/2}$ values markedly decreased, indicating a shortened life span. Temperature shift between 16°C and 25°C revealed that an increase in life span always involved low temperatures after the adult phase. High temperature treatment during the growing phase or after the adult phase caused an earlier start of the dying phase, but a downward temperature during the adult phase resulted in a great increase in the half-life of the population ($T^{1/2}$).

The results suggest that the life span of *C. elegans* is rigidly determined by somatic cells and markedly influenced by the effects of temperature on the cells during the post-mitotic state.

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Experimental Gerontology, Vol. 17, pp. 173–177, 1982
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AGE CHANGES IN THE H1 GROUP OF HISTONES FROM RAT LIVER

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(*Received* 27 *June* 1981)

INTRODUCTION

AGING HAS BEEN the subject of much speculation over the years and the sheer quantity of experimental data which has produced many conflicting results has led to considerable confusion.

The aging process is characterized by a declining ability of the individual to adapt to environmental stress and by an inability to maintain homeostasis.

It is generally believed that the biological aging process has a genetic determinant. In other words, aging is a part of the developmental program inscribed in the genetic material, DNA (Eichhorn, 1979; Morrow and Garner 1979). Hence, the attention paid to chromatin and its components: DNA, histones, and acidic proteins.

Since the appearance of the hypothesis about the repressive role of the histones in chromosomes (Alfrey *et al.* 1963; Stedman and Stedman, 1950), DNA-histone interactions have been a subject of interest to gerontologists (von Hahn, 1964, a, b).

The early excitement caused by the findings was soon moderated as crude preparations of histones could be fractionated into only five major classes, the proportions of which did not vary significantly in different tissues or different organisms. Thus, histones exhibit a uniformity and a lack of specificity which makes them appear to be involved in the structural organization of DNA into repeating subunits called nucleosomes (Kornberg, 1974; Thomas and Kornberg, 1975).

Of the five major classes, H1 appeared to be the most promising for aging research (for details, see Kanungo 1980).

In recent years, there have been a number of works which deal with age-related changes in histone composition but total agreement is still lacking because the source and conditions employed in the extraction and analysis of histones vary from work to work. Thus, Mitsui *et al.*, 1980, working with human diploid fibroblast cultures did find an age-related

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decline in histone H1 fractions, whereas Medvedev *et al.*, 1977, and Medvedev *et al.*, 1978, reported an age-associated increase in the histone fraction H1° and H1°(met) in the tissues from old mice and rats.

Gaubatz *et al.*, (1979a) requantitated the relative amounts of various histone fractions from mouse brain and liver finding no significant differences. The same conclusion was supported by the experiments of Carter and Chi-Bom (1975).

In the present paper we analysed the composition of histones extracted from rat liver in an attempt to obtain further information about eventual changes in histone composition in aging rat liver.

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METHODS

Animals

Each experiment was carried out on four groups of five Wistar male albino rats (young, 3–4 months; senescent, 24–28 months) maintained in a room at 22 ± 2° C on a 12 h light/12 h dark cycle. They were given commercial diet and water *ad libitum*. Rats were killed by cervical dislocation and their livers were removed, pooled, washed in physiological saline, and weighed.

Isolation of nuclei

The livers were homogenized by using a Teflon glass homogenizer, in 0.25 M sucrose-50 mM Tris-HCl buffer (pH 8.0)–0.1 mM phenylmethansulfonyl fluoride (Simpson and Sober, 1970). Nuclei and the cellular debris were sedimented by centrifugation at 1000 g for 10 min. The pellet was washed three times in 0.25 M sucrose-1 mM $MgCl_2$ – 50 mM Tris-HCl buffer, pH 8,–1% Triton X-100 and the purified nuclei were resuspended in 0.25 N HCl.

Isolation of histones

Crude histone was obtained by extracting the purified nuclei with 0.25 N HCl at +2° C for 18 hours (Chilina *et al.*, 1976). The acid extracted protein was precipitated in 15% trichloroacetic acid, washed with acified acetone, then with ether, and dried. The dry powder was dissolved in 15% sucrose 0.9 N acetic acid and used for electrophoresis after adjusting the concentration at 1 mg/ml using as a standard a 1 mg/ml aqueous solution of calf thymus histones (Calbiochem-Behring Corp., B grade). The amount of proteins was determined by the method of Lowry *et al.* (1951)

Electrophoresis of histones

Histones were fractionated on 0.5 × 15 cm, 15% acid urea polyacrylamide gels containing 6.25 M urea using the method of Panyim and Chalkley (1969). The gels were run at 2 mA/tube for 5 hours at +21° C, stained with 0.1% Amido black 10 B– 20% ethanol-7% acetic acid, and destained by several changes of 5% acetic acid. Finally, the gels were scanned at 560 nm by using a Carl Zeiss Jena densitometer.

RESULTS

As most of the workers involved in histone research are using polyacrylamide gel electrophoresis for histone analysis, we chose the same method described in detail by Panyim and Chalkley (1969). A typical densitogram is shown in Fig. 1. The ratio of the amounts of the major histone fractions from old and young rats was determined from the area under the peaks (Table 1).

As one can see from Fig. 1 and Table 1 there is a significant increase in the ratio of H1° in old animals as compared to young animals.

At the same time, there are also some changes in the ratio of H1, H3, H2a + H2b, and H4 in old rats as compared to young rats, but the values are not significant.

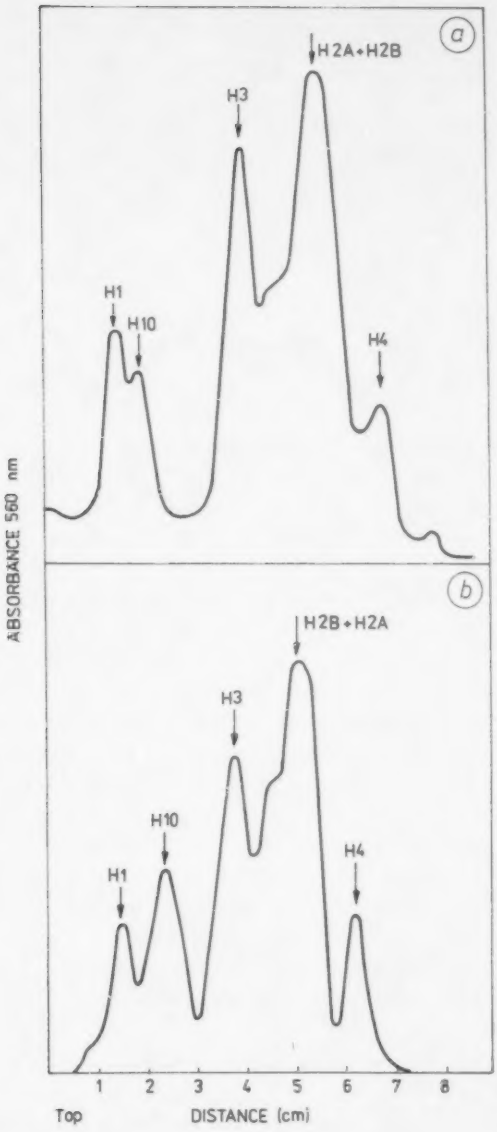

FIG. 1. Fifteen percent acid urea polyacrylamide gel electrophoretic patterns of 0.25 N HCl-extractable nuclear proteins of young(a) and old(b) rat liver. About 100 micrograms of protein were applied onto the gels and the electrophoresis was conducted for 5 hours at 2 mA/tube with a preelectrophoresis of 1 hour at +21°C. The proteins were stained with Amido black 10B.

DISCUSSION

Regardless of its location in chromatin, a striking feature of histone H1 is its variability among tissues and species (Kinkade, 1969; Bustin and Stollar, 1972; Bustin and Stollar, 1973). There are some possible functions attributed to H1 fraction in mammals. Thus, Brandburry *et al.* (1975) suggested that H1 could be involved in the formation of higher order structures of chromatin and Whitlock and Simpson (1976) suggest that histone H1 protects linker DNA against micrococcal nuclease digestion.

TABLE 1. RATIO OF AMOUNTS OF THE CORRESPONDING HISTONE FRACTIONS FROM OLD AND YOUNG RATS

Experiment No.	H1	H1°	H3	H2a + H2b	H4
1	0.89(4)[a]	2.44	1.08	1.01	1.00
2	1.22	4.05	1.04	0.73	1.35
3	0.80	4.40	0.77	0.74	1.40
4	0.92	2.45	0.53	1.05	0.97
Mean ± *S.D.*	0.95 ± 0.18	3.33 ± 1.03	0.85 ± 0.25	0.88 ± 0.17	1.18 ± 0.22
	NS	$P < 0.05$	NS	NS	NS

[a]Each value represents the mean and the figures in parentheses represent the overall number of determinations.

NS = nonsignificant.

The area under the peaks was calculated with the method of Mori *et al.* (1978) and the theoretical ratio of histone fractions was assumed to be 1.00.

Most important, it was found that the distribution of whole histone H1 among nucleosomes is uneven (Gaubatz and Chalkley, 1977; Albright *et al.*, 1979; Gorka and Lawrence, 1979).

In addition, the amount of H1 subfractions is a function of the size of the subunits, the greatest variation being observed for H1°° which was shown to be the most efficient in the protection of linker DNA (Gorka and Lawrence 1979). Hence, an increased resistance of linker DNA (that DNA which connects two adjacent nucleosomes) from old rats against digestion by micrococcal nuclease would be expected. This increased resistance would result in: (1) an increase in bandwidth of nucleosomal DNA on polyacrylamide gels upon chromatin digestion by micrococcal nuclease, in old animals, and (2) a decrease in fraction-containing nucleosomes (1SF) and an increase in fraction-containing oligonucleosomes (2SF) after chromatin digestion by the same enzyme in old animals (for technical details, see Tas *et al.*, 1980).

Recently, Gaubatz *et al.*, (1979b) found no marked differences between nucleosomes obtained by micrococcal nuclease digestion of young and old mouse chromatin extracted from heart and liver, but they did find an increase in bandwidth of the polyacrylamide gel electrophoretic bands of the nucleosomal DNA prepared from old mouse brain.

The second assumption is confirmed by the experiments of Tas *et al.* (1980) but they gave no satisfactory explanations for these results as their reagent, 2-mercaptoethanol, did not correct the ration 1SF/2SF in old mice.

As a conclusion, our experimental results support the findings of Medvedev *et al.* (1977) and Medvedev *et al.* (1978) rather than those of Carter and Chi-Bom (1975) and Gaubatz *et al.* (1979a), *i.e.* there is an increase in the histone H1° fraction in the livers of aged rats.

SUMMARY

Histones were obtained from young and old rat livers by extracting them in 0.25 N HCl.

They were fractionated on 15% acid urea polyacrylamide gels containing 6.25 M urea and the changes in the ratio of the major histone fractions as a function of age were calculated.

Data presented show a significant increase in the amount of H1° fraction in the liver of old rats as compared to young rats. This data is discussed and the possible involvement of H1° fraction in an increased resistance of old rat liver chromatin to micrococcal nuclease digestion of linker DNA is suggested.

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Finally, in connection with this increased resistance, some possible consequences in chromatin structure are discussed.

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AGE-RELATED CHANGES IN THE CORNEAL ENDOTHELIUM OF THE RAT

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(*Received* 25 *July* 1981)

INTRODUCTION

THE HUMAN corneal endothelium is a single layer of thin, mostly hexagonal cells. Mitosis occurs in the very young endothelial cell but is extremely rare in adult cells. In a study of corneas taken from 64 cadavers Irvine and Irvine (1953) found endothelial cell concentrations to vary from 1,281 per mm² to 4,153 per mm². These authors observed low cell concentrations to be accompanied by increased pleomorphism, but noted no association between cell concentration and age. Making use of the specular microscope, whereby the corneal endothelium can be examined and photographed in the living eye, Laing *et al.* (1975) and Bourne and Kaufman (1976) found, in populations of 61 and 40 subjects, respectively, statistically significant decreases in endothelial cells with age. Laule *et al.* (1978) studied 152 human subjects ranging in age from 3 to 88 years. They found aging changes in the endothelium to include loss of cells, increasing pleomorphism, and the development of apparent defects in the endothelial cell coverage of Descemet's membrane. Hoffer and Kraff (1980) documented decreasing cell numbers with age in 3,000 human eyes, but found the decrease so small that a large range was noted for each age. They concluded that age is not useful in estimating endothelial cell density. Except for these human studies, the corneal endothelium of animals nearing the end of their life spans has not been described. Our study of three ages of rats showed age-related changes in the cornea remarkably similar to those described for humans. Preliminary results suggest that mean endothelial cell area may prove to be a useful index of physiological age in experimental studies of aging.

MATERIALS AND METHODS

Fifteen female Sprague Dawley rats, five each of ages 6, 14, and 30 months, were obtained from the animal colony maintained by Charles River Breeding Laboratory for the National Institute on Aging. The median survival of female Sprague Dawley rats maintained in a barrier facility at Charles River Laboratories is 30 months,

This project was supported by a grant to A.R. from the National Institute on Aging #AG01548.

and maximum survival is 40 months (Adelman *et al.*, 1978). Rats were maintained in our facility for about two weeks.

The eyes from these rats were removed immediately after decapitation. The anterior one-third of the eye was excised and the central cornea cut into segments approximately 1 × 2 mm in a cold bath of phosphate buffered 5% glutaraldehyde. A 30-minute fixation in glutaraldehyde was followed by fixation in buffered 1% osmium tetroxide, dehydration in acetone and critical point drying using liquid carbon dioxide. The samples were then coated with a 20 nm thick layer of gold-palladium and examined in a Cambridge Stereoscan scanning electron microscope operating at 10 kV. Special care was taken to photograph all samples at 1000 X magnification.

We determined cell areas by precisely tracing the cell outlines on the photographs with a Numonics Model 1224 electronic digitizer. For statistical reasons, the numbers of cells counted in each photograph was limited to 12, since that was the largest number of whole cells traceable on the photographs of cornea from the 30-month old rats. The data were analyzed using nested analysis of variance and *t*-tests.

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RESULTS AND DISCUSSION

Figs. 1, 2 and 3 show scanning electron micrographs of the corneal endothelium of animals from each of the three age groups (animals 1, 2 and 6 of Fig. 4). The pattern of cell size and irregularity of cell borders found in the human eye is also clearly evident in the eye of the rat.

The frequency of microvilli per cell was unrelated to age. Some cells had no microvilli and some others as many as 90–100. This contrasts with a more consistent finding of 20–30 microvilli per cell in human (Hogan *et al.*, 1971) and rabbit (Blümcke and Morgan-roth, 1967) corneal endothelium. Fig. 4 shows the mean cell areas in μm^2 in each of the 15 animals.

It is apparent that the endothelium of the rat cornea shows many of the age-related changes found in the human eye. The process in humans of endothelial cell loss with age, and what we perceive as a compensatory spreading and thinning of surviving cells to cover the deficit, was also found to be present in the rat. However, a significant difference in mean cell area ($p < 0.001$) was found among rats of the same age. This would be expected if aging in general proceeds at individual rates.

The diminution in number of endothelial cells can be expressed either in terms of mean cell size or in terms of cells per square millimeter. In either instance, the similarities between rat and human are striking. The mean endothelial cell density in the human cornea has been reported to decrease from about 4,400 cells per mm^2 at age 3 to 2,850 cells per mm^2 at age 25, and to 2,077 cells per mm^2 at age 80 (Laule *et al.*, 1978). This reflects a steeper decline from ages 3 to 25, and a more gradual decline from ages 25 to 88. These figures correlate remarkably well with the mean endothelial cell densities of 3,891, 2,976, and 2,469 for 6, 14, and 30 month old rats, respectively. The calculated survival of endothelial cells after age 3 in humans was reported to be about 70% at age 25, and 53% at age 60 (Laule *et al.*, 1978). Our observations suggest that 30-month old rats have approximately 63% of the endothelial cells present at 6 months of age.

Mean endothelial cell areas have been reported for 61 humans ranging in age from 20 to 88 years (Laing *et al.*, 1976). It was found to approximately double from age 20 to age 80, from a mean cell area of about 400 μm^2 to nearly 800 μm^2. In the rats the mean endothelial cell area increased 1.6 times between the ages of 6 months and 30 months.

These data suggest that the corneas of 6, 14, and 30 month old rats correspond roughly to human corneas of approximately 20–30, 40–60, and 70–80 years of age. A decline in number of endothelial cells begins early in life in both species, and continues at steadily decreasing rates into very old age.

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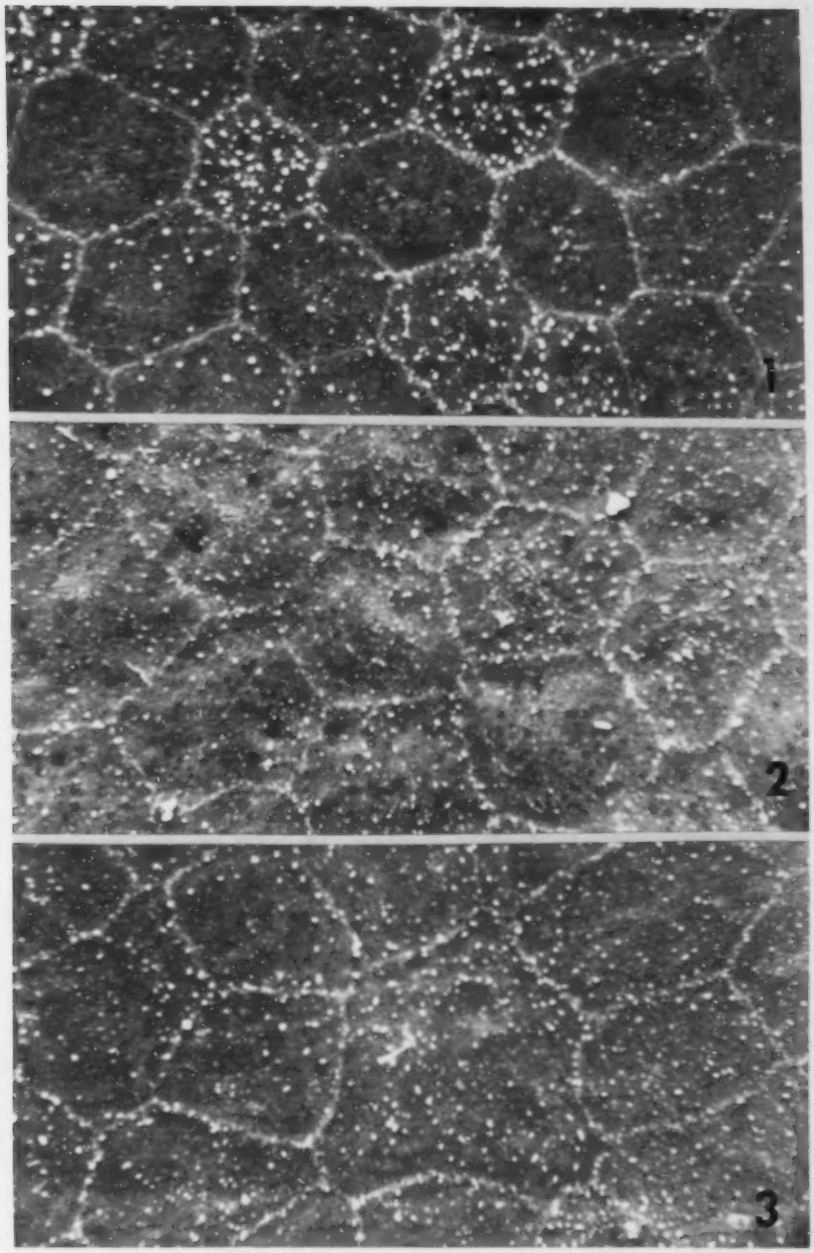

FIGS. 1, 2, and 3 are scanning electron micrographs of the corneal endothelium of three female Sprague Dawley rats aged 6 months, 14 months and 30 months, respectively (corresponding to animals 1, 2 and 6 in Fig. 4). Note enlargement of cells and distortions of the basic hexagonal form with increasing age. White dots are microvilli whose occurrence appears to be unrelated to age. All micrographs are of the same magnification (1400X).

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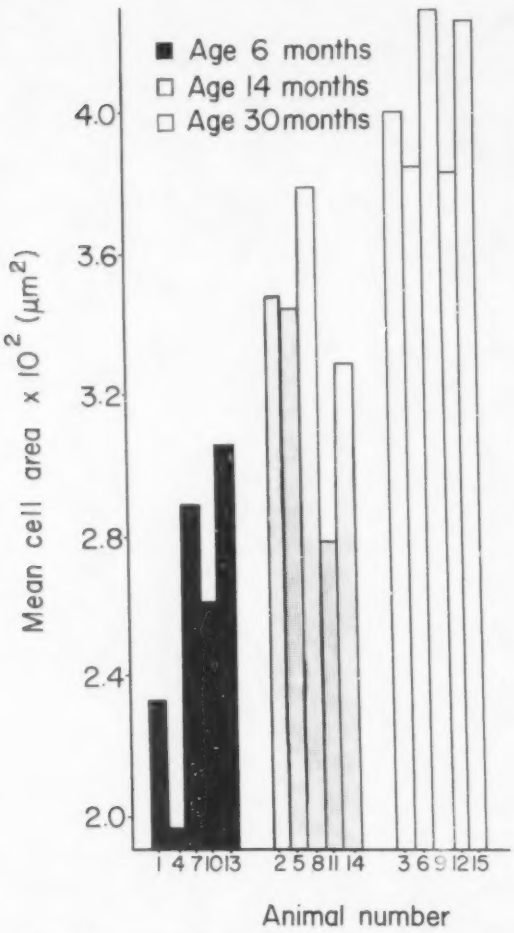

FIG. 4. Mean cell areas in μm^2 of 12 randomly selected cells in each of the 15 animals studied. The cell areas were calculated directly from the polaroid positives made from the scanning electron microscope at a magnification of 1000X. Nested analysis of variance and follow-up *t*-tests revealed significant differences among the three age groups ($p < 0.001$) and significant differences among animals within age groups ($p < 0.001$).

SUMMARY

The endothelial cells of the rat cornea show a progressive decline in numbers and an increase in pleomorphism from age 6 months to 30 months. These age-related changes correspond closely to those reported in the human cornea from approximately age 20 to age 70 years. The corneal endothelium may be found useful as an index of physiological aging in these and other mammals.

Acknowledgements—We thank Dr. Alan Katz for his assistance in statistical analysis and Drs. John Frehn and Edward Mockford for reading the manuscript.

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Experimental Gerontology, Vol. 17, pp. 185–194, 1982
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EFFECTS OF AGE AND DIABETES MELLITUS ON THE SOLUBILITY OF COLLAGEN FROM HUMAN SKIN, TRACHEAL CARTILAGE AND DURA MATER

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(*Received* 8 *August* 1981)

INTRODUCTION

COLLAGEN IS a major structural component in human beings, comprising about 30% of total body protein (Eyre, 1980). When an individual stops growing, the turnover rate of collagen becomes very low (Kivirikko, 1973). Thus, collagen is susceptible to changes that require a long period of time to occur.

Age-related changes in human collagen have been observed to include a decreased solubility (Bakerman, 1962), increased stiffness (Kohn, 1978) and an increased resistance to enzymatic digestion (Hamlin and Kohn, 1971; Zwolinski *et al.*, 1976; Hamlin *et al.*, 1978a,b). Data from animal studies, moreover, suggest that collagen from different tissues (McGavack and Kao, 1960; Schofield, 1980), as well as collagen from similar tissues but different animals (Hamlin *et al.*, 1980), may age at different rates. Although the precise chemical changes which collagen undergoes as a function of age are not known, the nature of the physical changes suggests a progressive crosslinking or stabilization (Heikkinen, 1973; Kohn, 1978; Bentley, 1979). Furthermore, collagen from individuals with diabetes mellitus appears to undergo premature aging, as manifested by greatly increased resistance to collagenase digestion (Kohn and Hamlin, 1978; Kohn and Schnider, in press). Moreover, many of the complications affecting diabetics resemble debilities of age and occur in collagen-rich tissues (Rosenbloom, 1977; Kohn and Hamlin, 1978). This suggests that a generalized accelerated aging of collagen in the diabetic may play an important role in the pathogenesis of such complications.

Recently, we have described several age-related changes in human collagen. These include a decreased solubility of skin collagen in acetic acid, less skin collagen released by pepsin digestion, increased nonenzymatic glucosylation of insoluble skin (Schnider and Kohn, 1981) and tendon collagen (Schnider and Kohn, 1980), as well as increased resistance of skin collagen to collagenase digestion (Kohn and Schnider, in press). These age-

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related changes occured to a much greater degree in young individuals with diabetes mellitus (Schnider and Kohn 1981; Kohn and Schnider, in press).

Skin is primarily composed of types I and III collagen, and cartilage contains type II collagen (Eyre, 1980). Although the specific type of collagen in dura is not known, it is likely that this tissue, like tendon, is composed primarily of type I collagen. The present study was undertaken to compare rates of aging of human collagen, as measured by solubility, in different tissues, and to determine if the apparent acceleration of aging in diabetes is generalized, in occuring at the different sites.

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MATERIALS AND METHODS

Subjects

Samples of abdominal or thoracic skin,[1] tracheal cartilage and dura mater were obtained at autopsy from 27 subjects without clinical or pathological evidence of connective tissue disease. Three of these subjects had juvenile onset diabetes (JOD) and three had maturity onset diabetes (MOD) as defined by criteria established by the National Diabetes Data Group (Harris and Cahill, 1979).

Preparation of tissues, extraction and assay procedures

Tissue samples were frozen at −70°C until used. Frozen samples were thawed and rinsed with tap water. Epidermis and subcutaneous fat were scraped from skin; perichondrium dissected from cartilage; and blood vessels dissected from dura. The remaining tissue was blotted dry, and approximately 0.75 g used for further processing, as previously described (Schnider and Kohn, 1981). Briefly, samples were homogenized in a neutral salt solution (1.0 M NaCl). Suspensions were stirred for 18 hours at 4°C and centrifuged. Collagen in the supernatant solution is referred to as the neutral salt soluble fraction.

The insoluble material remaining after the neutral salt extraction was extracted in an 0.5 M acetic acid solution with stirring for 18 hours at 4°C and centrifuged. The supernatant solution is referred to as acid-soluble collagen.

The insoluble material remaining after the acid extraction was suspended in 0.5 M acetic acid and digested with pepsin (Worthington Biochemicals, Freehold, NJ) at a concentration of 1.0 mg/ml for a final enzyme to substrate ratio of approximately 1:35, stirred for 18 hours at 4°C, and centrifuged. Collagen solubilized in this way is referred to as the pepsin-relased collagen fraction.

The collagen remaining after the two extraction procedures and the pepsin digestion is considered insoluble.

Collagen present in these fractions was estimated by measuring the amount of hydroxyproline. Hydroxyproline is assumed to comprise 14% of collagen by weight (Hamlin and Kohn, 1971). Replicate samples were hydrolyzed in 6.0 N HCl for 16 hours in an autoclave. The HCl was removed *in vacuo* and the amount of hydroxyproline determined by the method of Stegemann and Stalder (1967) as modified by Maekawa *et al.* (1970). The proportions of collagen in the four fractions were expressed as a function of age. Tissue samples were processed and all assays performed in replicate without knowledge of the age or clinical status of individual subjects.

Linear equations, regression coefficients and confidence bands were generated by a linear regression program with a calculator. Formulae for determining confidence bands were obtained from Armitage (1971). Probability values were determined from tables in Documenta Geigy (Diem, 1962).

RESULTS

The amount of collagen extracted from human skin, tracheal cartilage and dura mater by neutral salt and acetic acid solutions comprised a small fraction of the total collagen recovered. The greatest average amount of salt soluble collagen was recovered from skin. Cartilage contained a similar average amount of salt-soluble collagen, but dura had about one-third as much salt-soluble collagen as skin and cartilage. The greatest average amount of acid-soluble collagen also was recovered from skin, which contained about twice as much acid-soluble collagen as dura and six times as much as cartilage. Differences between these tissues in the amounts of recovered pepsin-released collagen were similar to

[1] The solubility data from human skin has been previously reported (Schnider and Kohn, 1981).

those found for the acid-soluble fraction. The greatest amount of collagen recovered from the tissues in the present study was insoluble.

There was a statistically significant age-related decrease in the proportion of neutral salt-soluble cartilage ($r = -0.589$, $P < 0.01$) and dura ($r = -0.51$, $P < 0.05$) collagen as shown in Fig. 1. There was also a suggestion of an age-related decrease in the proportion of neutral salt-soluble skin collagen ($r = -0.36$, $P > 0.05$). Two JOD had significantly less neutral salt-soluble cartilage ($P < 0.01$) and dura ($P < 0.05$) collagen, and one young MOD had significantly less neutral salt-soluble dura collagen ($P < 0.05$) than nondiabetics of similar ages.

The proportion of acid-soluble collagen (Fig. 2) was observed to decrease as a function of age for skin ($r = -0.44$, $P < 0.05$) and dura ($r = -0.49$, $P < 0.05$). The proportion of acid-soluble cartilage collagen, however, was low (usually less than 0.1%) and did not vary with age among nondiabetics ($r = -0.15$, $P > 0.10$). Two JOD had significantly less acid-soluble skin collagen ($P < 0.05$), but all three JOD had significantly less acid-soluble dura collagen ($P < 0.05$) than comparably aged nondiabetics.

The proportion of collagen solubilized by pepsin digestion was significantly decreased as a function of age for skin ($r = -0.809$, $P < 0.001$) and dura ($r = -0.74$, $P < 0.001$) but not cartilage, as shown in Fig. 3. The average proportion of collagen released from cartilage by pepsin digestion was low (2.3%) and remained relatively constant after 25 years of age. All JOD and one young MOD had significantly less collagen solubilized from skin ($P < 0.001$) and dura ($P < 0.005$) by this digestion procedure than comparably aged nondiabetics. The low amounts of pepsin-released collagen from cartilage samples precluded valid statistical comparisons between diabetics and nondiabetics. The proportion of collagen released from samples of older MOD skin, cartilage and dura by this digestion procedure did not differ from similarly aged nondiabetics.

The proportion of insoluble collagen remaining after the solubilization procedures increased linearly as a function of age in skin ($r = 0.804$, $P < 0.001$) and dura ($r = 0.76$, $P < 0.001$) but remained relatively constant after the 25 years of age in cartilage, as shown in Fig. 4. All JOD had significantly more insoluble skin ($P < 0.001$) and dura collagen ($P < 0.01$) than would be predicted for their ages. One young MOD had significantly more insoluble skin ($P < 0.001$) as well as an increased proportion of insoluble dura collagen. Two JOD also had more insoluble cartilage collagen than comparably aged nondiabetics. The two older MOD did not have values exceeding those predicted for nondiabetics of comparable age.

DISCUSSION

Skin from one 48-year-old nondiabetic subject had substantially more pepsin-released collagen and less insoluble collagen than other similarly aged nondiabetics (Fig. 3). This subject's clinical history was significant for undifferentiated sarcoma, which was irradiated. One possible explanation, as previously suggested (Schnider and Kohn, 1981) for the excessive solubility of his skin, may be that the skin used in this study was irradiated during treatment for the sarcoma. Collagen from irradiated areas is actinically damaged and known to be more soluble (Sams and Smith, 1965). This hypothesis is supported by the observation in the present study that this subject's cartilage and dura were not more soluble than other nondiabetics of the same age (Fig. 3).

The proportion of collagen extracted from human skin, tracheal cartilage and dura mater by neutral salt and acetic acid solutions comprised a small fraction of the total col-

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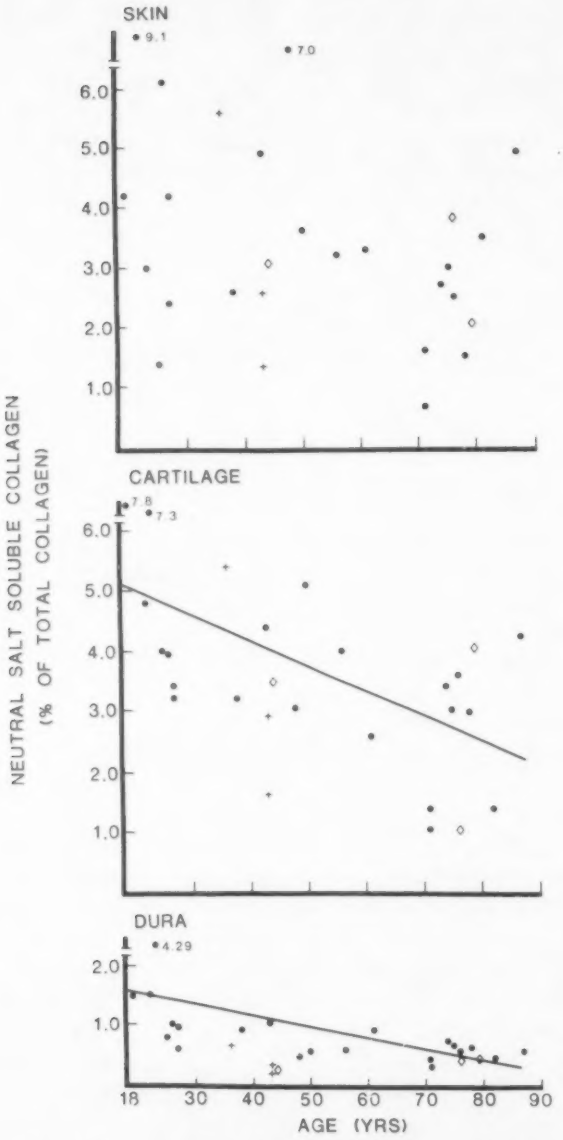

FIG. 1. The proportion of skin (a), cartilage (b) and dura mater (c) collagen extracted by 1.0 M neutral NaCl is plotted as a function of subject's age. The solid line in (b) represents the regression equation: $y = -0.042\,(\times) + 5.834$, $r = -0.589$, $P < 0.01$; and in (c) represents the regression equation: $y = -0.018\,(\times) + 1.87$, $r = -0.51$, $P < 0.05$. Both lines are derived from the data of nondiabetics. No confidence bands are plotted because of the large variability among young mature subjects. Skin solubility was not significantly related to subject age ($r = -0.36$, $P > 0.05$). Nondiabetic subjects (•, $n = 21$); Juvenile Onset Diabetics (+, $n = 3$) and Maturity Onset Diabetics (◇, $n = 3$).

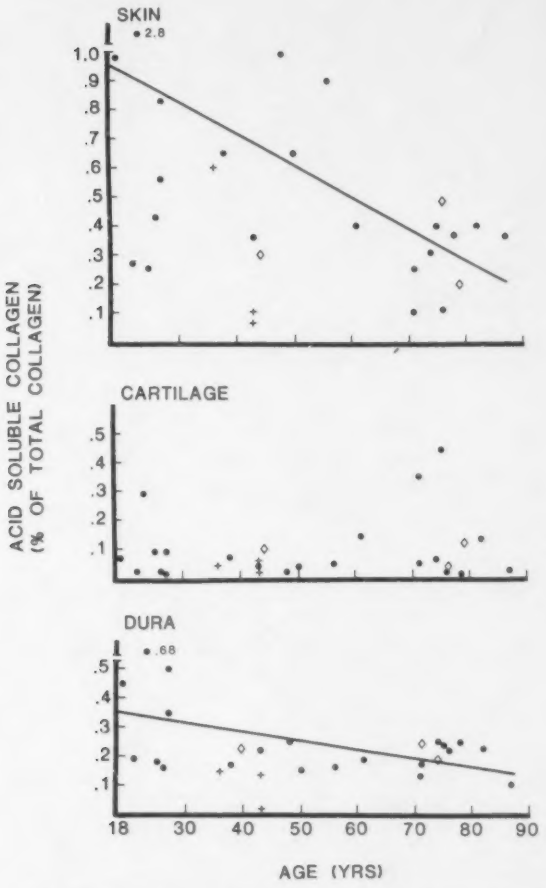

FIG. 2. The proportion of human skin (a), cartilage (b) and dura mater (c) collagen extracted by 0.5 M acetic acid is plotted as a function of subject's age. The solid line in (a) represents the regression equation: $y = -0.0107\ (\times) + 1.14$, $r = -0.44$, $P < 0.05$; the solid line in (c) represents the regression equation: $y = -0.003\ (\times) + 0.398$, $r = -0.49$, $P < 0.05$, and is derived from the data of nondiabetics. No confidence bands are plotted because of the large variability among young mature subjects. The proportion of acid-soluble cartilage collagen did not vary as a function of age ($r = -0.15$, $P > 0.10$). The symbols are the same as in Fig. 1.

lagen recovered. This probably reflects the slow rate of collagen synthesis following cessation of growth (Kivirikko, 1973; Kohn, 1978; Bornstein and Traub, 1979). An age-related decrease in the proportion of neutral salt-soluble cartilage and dura collagen was observed. There was a suggestion of an age-associated decrease in the proportion of neutral salt-soluble skin collagen but the decrease was not statistically significant. Previous studies have also observed that the proportion of neutral salt-soluble collagen does not

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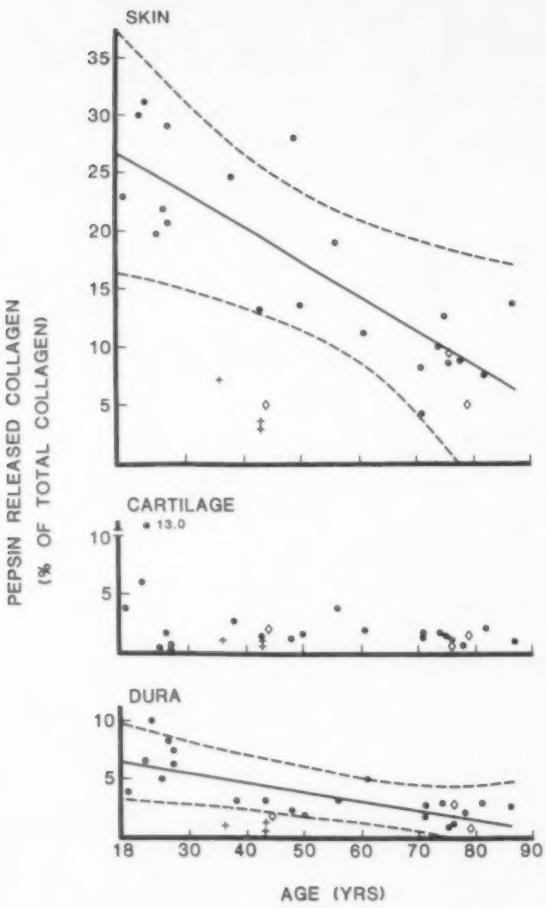

FIG. 3. The proportion of collagen released after pepsin digestion from human skin (a), tracheal cartilage (b) and dura mater (c) is plotted as a function of subject's age. The solid lines represent the regression equations in (a): $y = -0.295\ (\times) + 32.04$, $r = -0.809$, $P < 0.001$; and in (c): $y = -0.082\ (\times) + 8.085$, $r = -0.74$, $P < 0.001$. The proportion of pepsin-released cartilage collagen did not vary with age in (b) ($r = -0.38$, $P > 0.05$). The 99.5% confidence bands are represented by the dashed lines in (a) and (c). The regression lines and confidence bands are derived from the data on nondiabetics. The symbols are the same as in Fig. 1.

decrease as a function of age in human (Uitto *et al.*, 1971), mouse (Schofield, 1980) and guinea pig skin (Wirtschafter and Bentley, 1962).

Collagen is solubilized by neutral salt solutions from 0.15 to 1.0 M predominantly by disruption of noncovalent bonds. The neutral salt-soluble fraction of collagen is considered to represent the most recently synthesized fraction of collagen (Bornstein and

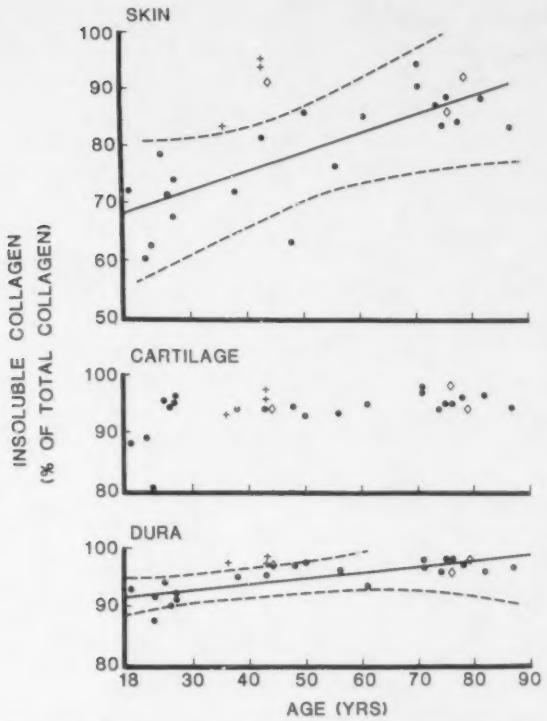

FIG. 4. The proportion of insoluble collagen remaining after the salt and acid extractions and pepsin digestion procedures from human skin (a), tracheal cartilage (b) and dura mater (c) is plotted as a function of subject's age. The solid lines represent the regression equations in (a): $y = 0.412\,(\times) + 57.94$, $r = 0.804$, $P < .001$; and in (c): $y = 0.096\,(\times) + 90.11$, $r = 0.76$, $P < 0.001$. The proportion of insoluble cartilage collagen did not vary with age in (b) ($r = 0.39$, $P > 0.05$). The 99.5% confidence bands in (a) and the 99% confidence bands in (c) are represented by the dashed lines. The regression lines and confidence bands are derived from the data on nondiabetics. The symbols are the same as in Fig. 1.

Traub, 1979). Thus, the turnover of human dura collagen was greater than cartilage and both decreased as a function of age. Although the average turnover of skin collagen was similar to that of cartilage, the turnover of skin collagen did not diminish significantly with age. If age-related changes in the properties of collagen and collagen-rich tissues are determined by the proportion of aged collagen, then a tissue with a constant supply of newly synthesized collagen might be expected to age at a slower rate than a tissue in which collagen synthesis remains at a low level or decreases as a function of age.

Extraction of collagen by acetic and citric acids is thought to occur predominantly by disruption of noncovalent interactions and covalent but not stabilized aldimine crosslinks. Collagen solubilized in this way is considered to represent a more mature fraction of collagen (Bornstein and Traub, 1979). The proportion of collagen solubilized by acetic acid

from human skin and dura was observed to decrease as a function of age. The proportion of acid-soluble cartilage collagen was usually low and did not vary with age. Age-related decreases in acid soluble collagen have also been reported in human (Bakerman, 1962) and mouse skin (Maekawa *et al.*, 1970; Schofield, 1980).

Collagen from human skin and dura became increasingly resistant to the solubilizing effects of pepsin digestion as a function of age. Amylase treated tendon collagen from a 65-year-old man has been reported to be one-tenth as soluble after pepsin digestion as tendon collagen from a 24-year-old man (Steven, 1966). Likewise, collagen from human tendon (Hamlin and Kohn, 1971), dura (Hamlin *et al.*, 1978a), myocardium (Zwolinski *et al.*, 1976) and skin (Kohn and Schnider, in press) has been shown to become progressively resistant to digestion by collagenase with advancing age.

Although human cartilage collagen did not show an age-related resistance to pepsin digestion, there appeared to be a marked resistance to the solubilizing effects of pepsin which remained constant after the age of 25. Cartilage collagen is immersed in a thick matrix of glycosaminoglycans (GaG) (Jackson and Bentley, 1968), and interactions between GaG and collagen might inhibit the solubilizing effect of pepsin digestion. Removal of GaG by chondroitinase ABC digestion or by guanidine-HCl extraction has been reported to markedly increase the solubilizing effect of pepsin digestion on bovine cartilage (Etherington, 1977). However, guanidine-HCl extraction of normal human cartilage did not result in increased solubilization (Herbage, 1975). Therefore, a role for GaG in the solubility of human cartilage appears unlikely. Insolubility is more likely to depend on interactions between collagen molecules. The marked degree of insolubility and presumed lack of turnover of cartilage collagen might indicate that articular cartilage collagen is exposed to life-long stress and trauma that contribute to development of degenerative joint disease (Hall, 1976; Schofield and Weightman, 1978).

The solubilizing effects of pepsin are thought to be primarily due to cleavage of the nonhelical regions of the collagen molecule. These regions are known to contain intramolecular crosslinks (Weiss, 1976). The nonhelical regions are also thought to contain intermolecular crosslinks, but this has not been proven (Bornstein and Traub, 1979). The largest proportion of collagen recovered from human skin, cartilage and dura mater, in the present study, was insoluble. An age-related increase in the proportion of insoluble skin and dura collagen but not cartilage collagen was observed. A suggestion of an age-related increase in the insoluble fraction of costal but not articular cartilage has been reported (Miller *et al.*, 1969). Collagen from human skin (Bakerman, 1962) and rat skin and tendon (McGavack and Kao, 1960) also have been reported to become insoluble as a function of age. It should be pointed out that changes in the solubility of collagen from a tissue may not be a sufficient measure of aging. Thus, the lack of an age-related increase in insoluble tracheal cartilage and articular cartilage (Miller *et al.*, 1969) collagen does not mean that cartilage collagen does not age. It is likely that insoluble cartilage collagen continues to age and may even do so more quickly than skin or dura. Insoluble skin collagen continues to age, as manifested by increasing resistance to enzymatic digestion (Kohn and Schnider, in press).

Although it has been postulated that the marked insolubility of collagen that occurs as a function of age is due to progressive stabilization or crosslinking of the collagen macromolecule, the precise chemical mechanism of this stabilization is not known (Heikkinen, 1973; Bailey *et al.*, 1974; Hall, 1976; Kohn, 1978; Hamlin *et al.*, 1978a,b; Bentley, 1979).

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The reducible crosslinks of human and other animal collagens, for example, have been shown to increase during growth. However, these crosslinks decrease to a very low level by maturity (Bailey and Shimokomaki, 1971). It was speculated that the decrease in reducible crosslinks was due to stabilization *in vivo*. Postulated mechanisms for *in vivo* stabilization include Amadori rearrangement (Bailey *et al.*, 1974), *in vivo* reduction (Mechanic *et al.*, 1971) oxidation (Bailey *et al.*, 1974), and by electrophilic addition (Davis *et al.*, 1975). These postulated stabilized crosslinks, however, have either not been isolated or if isolated have not been shown to increase with age. A nonreducible crosslink, formed by covalent association of three lysine or hydroxylysine residues, has also been reported. While an age-related increase in this crosslink was observed in rat cartilage and tendon, no such age-related change was observed with human cartilage or tendon (Moriguichi and Fujimoto, 1978). One possible mechanism for the age-related stabilization of human collagen is suggested by studies on the nonenzymatic glucosylation of human collagen.

Human skin and tendon collagen becomes progressively glucosylated as a function of age (Schnider and Kohn, 1980, 1981). Although the precise effect of glucosylation of collagen is not known, glucosylation of other proteins has been reported to cause crosslinking, as for example, between albumin molecules (Day *et al.*, 1980) and between human lens crystallin protein molecules (Monnier and Cerami, 1981). Thus, if glucosylation of human collagen causes crosslinks to form between collagen molecules, and glucosylation occurs as a part of the normal aging process, then it could play an important role in the age-related stabilization of collagen. Furthermore, in the context of this hypothesis, early maximal glucosylation of collagen could explain the premature age changes observed in collagen from individuals with diabetes mellitus.

SUMMARY

The solubility of collagen from human skin, tracheal cartilage and dura mater was compared. Skin contained the greatest amount of soluble collagen followed by dura and cartilage. An age-related decrease in the proportion of neutral salt-soluble collagen was observed for cartilage and dura. The proportion of collagen soluble in acetic acid decreased as a function of age in skin and dura. An age-related decrease in the proportion of skin and dura collagen released by pepsin digestion was also observed, as was a concomitant increase in the proportion of insoluble collagen in these tissues.

Individuals with juvenile onset diabetes had significantly less salt-soluble dura collagen, acid-soluble skin and dura collagen, pepsin-released skin and dura collagen, and significantly more insoluble skin and dura collagen than comparably aged nondiabetics. One young maturity onset diabetic had significantly less salt-soluble dura collagen, pepsin-released skin and dura collagen, and significantly more insoluble skin and dura collagen than similarly aged non-diabetics. The solubility of collagen from tissues of older maturity onset diabetics, however, did not differ significantly from other old nondiabetics.

These data indicate that there is a direct effect of age on the solubility of collagen from human tissues, and that based on solubility, cartilage collagen ages faster than dura collagen, with skin undergoing the slowest relative aging. These data also support previous observations indicating that collagen from individuals with diabetes mellitus undergoes accelerated aging. Alterations in collagen may be responsible for some of the debilities of age that occur in collagen-rich tissues, and which appear at an earlier age and more severely in individuals with diabetes mellitus.

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Experimental Gerontology, Vol. 17, pp. 195–203, 1982
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POST-NATAL DEVELOPMENTAL CHANGES IN MYOCARDIAL CONTRACTILITY OF THE FEMALE WISTAR RAT

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(*Received* 4 *September* 1981)

Abstract—The intrinsic contractile properties of papillary muscles isolated from female Wistar rats during post-natal development were examined. In addition, the myocardial responsiveness to certain inotropic interventions was studied. In muscles from 60-day-old animals, the velocity of shortening was markedly greater than muscles from animals ≥ 180 days old. These findings occured in the presence and absence of added norepinephrine or calcium and over a wide range of contraction frequency. No major age-related differences in responsiveness to the various inotropic agents was observed. Isometric tension was significantly higher in muscles from animals ≥ 180 days of age when compared to 60-day-old rats. Thus, post-natal development profoundly affects the intrinsic contractile state of cardiac muscle, with the force of contraction increasing and the speed of shortening decreasing as animals age.

INTRODUCTION

AGING RESULTS in profound changes in cardiovascular function (Shreiner *et al.*, 1969; Lee *et al.*, 1972; Rothbaum *et al.*, 1973). Cardiac index, heart rate, mean systolic ejection rate and the response to sympathetic stimuli and cardiac glycosides are reduced, while peripheral vascular resistance, mean diastolic and systolic pressure and arterio-mixed venous O_2 difference are elevated during aging (Landowne *et al.*, 1955; Harrison *et al.*, 1964; Solomon *et al.*, 1965; Weisfeldt *et al.*, 1971a; Lakatta *et al.*, 1975a; Lakatta *et al.*, 1975b; Guarnieri *et al.*, 1979). These alterations in cardiovascular function have been interpreted as secondary to changes in enzyme structure and function (Syrovy and Gutman, 1979; Kanungo, 1975; Kanungo and Patnaik, 1972; Capasso and Zimmerman, 1980), in oxygen delivery (Weisfeldt *et al.*, 1971; Rakusan and Poupa, 1964; Gold *et al.*, 1968; Abu-Erreish *et al.*, 1977), in peripheral resistance (Rothbaum *et al.*, 1973; Friedman *et al.*, 1958; Pomerance, 1965) and in the autonomic nervous sytem (Sonnenblick, 1968; Conway *et al.*, 1971; Finch, 1972; Finch, 1973) induced by senescence.

Papillary muscles isolated from the left ventricle of aged rats and studied isometrically have shown an increased duration of isometric contraction (Lakatta *et al.*, 1975a), a pro-

Supported in part by NIH grant #HL 21993-02 and #HL 07071-05

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longation of time to peak tension (Lee *et al.*, 1972; Lakatta *et al.*, 1975c) and no difference in peak developed tension (Weisfeldt *et al.*, 1971b). Isotonically, the velocity of muscle shortening (*Vs*) of left ventricular papillary muscles from adult rats decreases as a direct function of age (Alpert *et al.*, 1967).

However, the possibility exists that post-natal development exerts an effect on the intrinsic contractile properties of the myocardium. Developmental changes and their contribution to age-related cardiovascular alterations have not been clearly defined (Shreiner *et al.*, 1969; Lee *et al.*, 1972; Weisfeldt *et al.*, 1971a; Lakatta *et al.*, 1975a; Spurgeon *et al.*, 1977). Furthermore, the age-related mechanical response to norepinephrine is essential in relation to contractility, since considerable evidence indicates a diminished inotropic response of the aged myocardium to catecholamines (Lakatta *et al.*, 1975a; Guarnieri *et al.*, 1979; Gey *et al.*, 1965).

Accordingly, the aim of the present investigation was to determine the effects of development on: (1) the intrinsic contractile properties of the myocardium and (2) the contractile response of the myocardium to various inotropic stimuli. The development of methods for detailed and quantitative analysis of mechanical characteristics of isolated papillary muscles (Sonnenblick, 1962), removed from the nervous, humoral, and metabolic influences that exist *in vivo*, and the ability to compare the function of muscle from different groups of animals (Spann *et al.*, 1966), permit critical examination of these problems.

The basic experimental plan was to compare the behavior of muscles from female Wistar rats at 60, 120, 180, 240 and 300 days of age. The intrinsic contractile properties of isolated heart muscle were examined in papillary muscle and the effects of certain inotropic interventions were studied.

The results of these studies indicated that although the intrinsic contractile state of cardiac muscle is affected, its responsiveness to inotropic agents remains unchanged during post-natal development.

MATERIALS AND METHODS

Animal model

Female Wistar rats at 4 weeks of age (75 g) were stored in our animal facility on a 12 hour light–12 hour dark cycle. Animals were fed Purina® rat chow and water *ad libitum*. Animals were then studied at 60, 120, 180, 240 and 300 days of age.

Instrumentation and experimental design

At the time of study, the heart was quickly removed after administration of ether anesthesia. The papillary muscle was rapidly dissected from the left ventricle and suspended vertically in a myograph mounted on a Palmer stand. The tendinous end was tied with Ethicon 3–0 braided silk to a light weight stainless steel rod which was inserted into a magnesium lever (equivalent mass of 125 mg) and held in position by an upper micrometer stop. The bathing solution was a modified Krebs-bicarbonate buffer containing, in millimoles per liter: Na 140.2, K 3.5, Mg 1.1, Cl 119.0, HCO_3 24.8, H_2PO_4 1.1, SO_4 1.1, Ca 0.6 and dextrose 5.5. The solution was maintained at a temperature of 27 ± 1°C and continuously aerated with 95% O_2–5% CO_2 which yielded a pH of 7.4. The muscle was stimulated with rectangular pulses 15 msec in duration and 20% above threshold at a frequency of 0.1 Hz, provided through platinum electrodes arranged on either side of and parallel to the muscle preparation.

The parameters of muscle force and its first derivative, muscle length and velocity, were displayed as a function of a time on a multichannel storage oscilloscope (Tektronix, 5103N). Force was measured by a strain gauge transducer (DSC-3, Kistler-Morse Corp.) attached via a spring loaded clip to the non-tendinous end of the muscle. Velocity was determined by passing the muscle length signal (lever position) through an active analog dif-

ferentiator (time constant ± 0.05 msec). The rate of tension development was derived from differentiation of the force signal.

After an equilibration period of 90 min, during which the muscle contracted isometrically with a resting force of 1 gram, the length-tension curve was generated by reducing muscle length from the length associated with a maximum developed force (*Lmax*) to about 88% Lmax in 0.1 mm steps, and the resting and developed force (and its differential) were recorded. The relationship between force and velocity for a series of afterloaded contractions was established at an initial muscle length of *Lmax*. The load on the muscle was progressively increased from preload to isometric force by varying the current passed through a coil suspended by bearings in a magnetic field, and the resulting shortening and relaxation velocities were measured.

At the completion of each experiment, the muscle length was measured, and the muscle was blotted dry and weighed. The cross-sectional area of the muscle was calculated by dividing its weight by its length, assuming the geometry of a cylinder and a specific gravity of 1.

Interventions

The effects of several interventions on contraction and relaxation processes were examined: (1) the frequency of stimulation was increased from 0.1 Hz to 0.8 Hz by successive doublings of rate every 2 min; (2) calicum concentration was increased from 0.6 to 2.4 mM, and (3) norepinephrine (Levophed, Winthrop) was added to the bathing solution in tenfold increments, increasing concentrations from 10^{-8} M to 10^{-5} M at 15 min intervals. In addition to increasing calcium to 2.4 mM no more than one other intervention was performed on each papillary muscle.

Data analysis

Values obtained from mechanical determinations are presented as mean ± standard error of the mean. Mean values between age groups and the response to experimental interventions were compared by multifactor analyses of variance followed by multiple contrasts with the Student's *t*-test. Differences with p values ≤ 0.05 were considered significant.

RESULTS

Analysis of isotonic contractions

Velocity of isotonic shortening (*Vs*) at a given relative load (total isotonic load/total isometric load × 100) was found to vary inversely with age (Fig. 1 and Table 1). At the lightest relative load studied (20%), *Vs* averaged 1.41 ± 0.03 muscle lengths/sec in the muscles from 60-day-old rats and was significantly lower in the muscles obtained from 180-day-old rats (1.21 ± 0.02 muscle lengths/sec). In addition, the *Vs* in the muscles from 300-day-old rats (1.13 ± 0.02 muscle lengths/sec) was significantly lower than that in the muscles from 180 day old rats.

Analysis of isometric contractions

The compliance of the papillary muscle was unchanged during development as shown by the essentially identical length-resting tension curves in the muscle from all five groups of animals (Fig. 2). At *Lmax*, i.e., the muscle length at which active tension was maximal, resting tension averaged 1.9 ± 0.6 g/mm² in 180-day-old rats with no significant differences in the muscle from the other four groups of rats. These values did not differ significantly from one another nor were there differences in resting tension among the five groups of muscles at other portions of the length-resting tension curve.

Active tension at the peak of the length-active tension curve averaged 4.2 ± 0.4 g/mm² in the muscles from 60-day-old rats, 5.6 ± 0.5 g/mm² in the muscles from 180-day-old rats and 7.3 ± 0.3 g/mm² in the muscles from 300-day-old rats (Table 1). Thus, active tension showed a significant increase from 60 to 180 days of age and again from 180 to

TABLE 1. GENERAL FEATURES OF POST-NATAL DEVELOPMENT IN RATS.

Age (days)	60 (N = 12)		120 (N = 11)		180 (N = 10)		240 (N = 11)		300 (N = 12)
BW (g)	210 ± 6.0		258 ± 3.0		261 ± 5.0		273 ± 6.0		280 ± 5.0
p		≤0.01		NS		NS		NS	
HW (g)	0.53 ± 0.02		0.63 ± 0.01		0.64 ± 0.02		0.66 ± 0.02		0.68 ± 0.02
p		≤0.05		NS		NS		NS	
DT (g/mm²)	4.2 ± 0.4		4.3 ± 0.4		5.6 ± 0.5		7.1 ± 0.4		7.3 ± 0.3
p		NS		≤0.05		≤0.05		NS	
TPT (msec)	113 ± 1.0		115 ± 1.0		121 ± 2.8		120 ± 1.6		123 ± 2.0
p		NS		≤0.05		NS		NS	
$T½R$ (msec)	85 ± 3.0		86 ± 3.0		106 ± 6.0		120 ± 3.0		127 ± 2.0
p		NS		≤0.05		≤0.05		≤0.05	
$+T'$ (g/sec/mm²)	114 ± 6.3		111 ± 5.9		108 ± 6.8		103 ± 4.1		99 ± 4.1
p		NS		NS		NS		NS	
$-T'$ (g/sec/mm²)	55 ± 4.8		54 ± 2.4		53 ± 4.0		51 ± 1.5		50 ± 1.3
p		NS		NS		NS		NS	
Vs (ML/sec)	1.41 ± 0.03		1.38 ± 0.02		1.21 ± 0.02		1.18 ± 0.02		1.13 ± 0.02
p		NS		≤0.05		NS		≤0.05	

BW = body weight; HW = heart weight; DT = developed tension; TPT = time to peak tension, $T½R$ = time to ½ relaxation (50% DT) $+T'$ = peak rate of tension rise; $-T'$ = peak rate of tension fall; Vs = peak velocity of shortening divided by $Lmax$; NS = not significant ($p \leq 0.1$).

Values are expressed as the ± SEM. Number of animals are given in parentheses at the top of each column. Vs values are obtained at an initial muscle length = $Lmax$, and at a relative load (total isotonic load/total isometric load × 100) of 20%.

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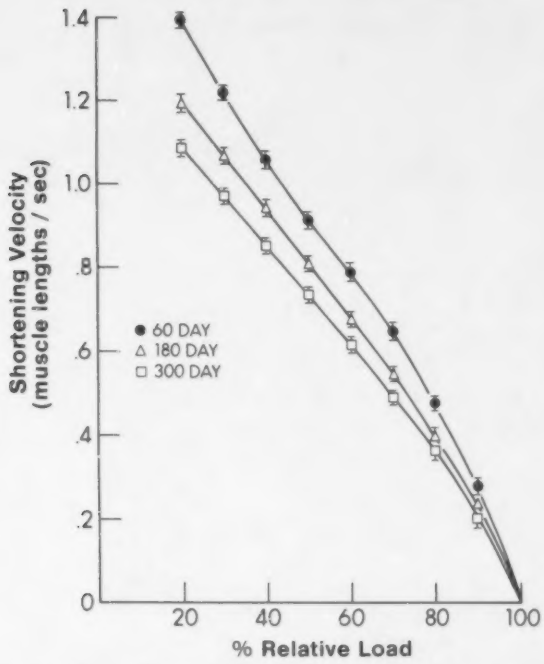

FIG. 1. Normalized force-velocity relations at a bath Ca++ = 2.4 mM. Values were obtained from a series of afterloaded isotonic contractions at an initial muscle length of Lmax. Force is expressed as the percent relative load (total isotonic load/total isometric load × 100). Velocity is expressed as the number of muscle lengths per second, calculated at the peak velocity (in mm/sec), divided by Lmax. Number of animals noted in Table 1. Values are plotted as the mean ± SEM. Significant differences are present between all groups at relative loads ≤ 75%. Data from 120 and 240-day-old animals not significantly different from 60 and 300-day-old animals respectively.

300 days of age. Active tension from muscles of 120 and 240-day-old rats was not significantly different than from muscles of 60 and 300-day-old rats respectively.

When tension was explained as a function of time and analyzed in terms of the rate of tension development and contraction duration, it was observed that aging affects the contractile performance of the isometrically contracting muscle to a great extent (Fig. 2 and Table 1). Time to peak tension (*TPT*) and time to 1/2 relaxation (*T1/2R*) varied directly with the age of the rats while the peak rate of tension rise (+*T*) and the peak rate of tension fall (−*T*) revealed similar, although less dramatic, changes (Table 1).

Frequency response

Variations in the frequency of contraction from 6 to 48 per min resulted in directionally similar changes in the time to peak tension and the rate of tension development in muscles from each group of animals (Fig. 3a,b). Consequently the difference in time to peak ten-

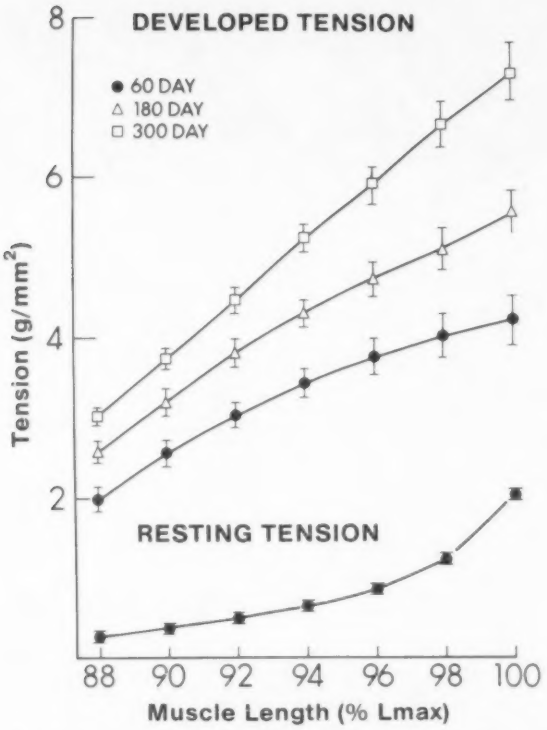

FIG. 2. Resting tension (*RT*) and developed tension (*DT*) are plotted as a function of muscle length in left ventricular papillary muscles from 60, 180 and 300-day-old female Wistar rats. Number of animals noted in Table 1. Values are presented as the mean ± SEM. Significant differences in *DT* are present in all groups at all muscle lengths studied. Data from 120 and 240-day-old animals not significantly different from 60 and 300-day-old animals respectively. No significant age-related differences in *RT* were noted.

sion and the rate of tension development observed at six contractions per min among the five age groups pertained to the entire range of frequency studied.

Absolute tension represents the interaction between the rate of tension development and the time to peak tension. When frequency of contraction was increased from 6 to 48 per min, the time to peak tension declined (Fig. 3a) while the rate of tension development changed little in all five groups of muscles (Fig. 3b). In the muscles from all groups the time to peak tension declined proportionately more than the rate of tension development, and, as a consequence, tension decreased as frequency was elevated (Fig. 3c).

Responsiveness to norepinephrine

The increase in the velocity of shortening observed after the addition of norepinephrine was similar in muscles from all groups of animals (Fig. 4a). At the lowest relative load studied (20%), *Vs* averaged 1.69 ± 0.04 muscle lengths/sec in 60-day-old animals at a

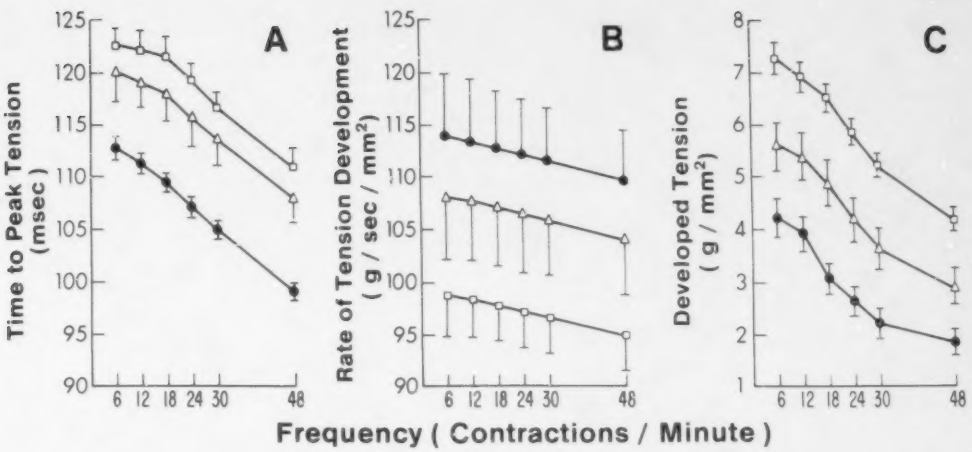
A
B
C
Time to Peak Tension (msec)
Rate of Tension Development (g / sec / mm²)
Developed Tension (g / mm²)
Frequency (Contractions / Minute)

FIG. 3. Time to peak tension (A), rate of tension development (B) and developed tension (C) measured at various contraction frequencies in muscle from 60-day-old rats (•, $N = 12$), 180-day-old rats (△, $N = 10$) and 300-day-old rats (□, $N = 12$). Values are plotted as the mean ± SEM. Significance for the entire range of frequencies remains as in Table 1. Data from 120 and 240-day-old animals not significantly different from 60 and 300-day-old animals respectively.

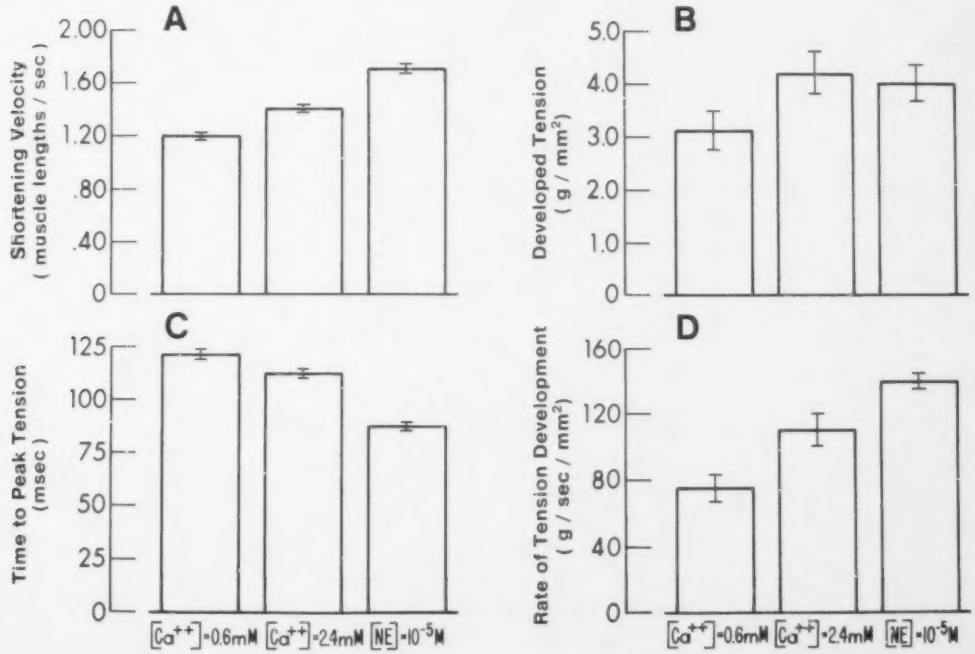

FIG. 4. Shortening velocity (A), developed tension (B), time to peak tension (C) and the rate of tension development (D) measured at an external bath calcium concentration of 0.6 mM and 2.4 mM and upon the addition of norepinephrine (10^{-5} M) to muscles equilibrated at a bath [Ca++] = 2.4 mM. Data shown is from 60-day-old female Wistar rats. Relative changes were similar in the remaining four groups. Significant differences are present among all calcium and norepinephrine levels with the exception of developed tension between [Ca++] = 2.4 and NE = 10^{-5} M.

norepinephrine concentration of 10^{-5}. This represented a 20% increase in *Vs* upon the addition of norepinephrine. Similar increases were found in the remaining four age groups. Although developed tension showed a slight decrease at a norepinephrine concentration of 10^{-5} M, significant reduction of the time to peak tension and augmentation of the rate of tension development were observed in all five groups (Fig. 4b, c, d). Thus, in 60-day-old rats, time to peak tension averaged 88 ± 1.3 msec and the rate of tension development averaged 140 ± 3.1 g/sec/mm² at a norepinephrine concentration of 10^{-5} M.

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Calcium responsiveness

The positive inotropic effect of increasing external calcium from 0.6 mM to 2.4 mM is seen in Fig. 4a–d. Although speed of contraction and force development both increase with an increase of external calcium, no noticeable differences were observed among the muscles from the five different groups of rats. Thus, force increased 25% in all animals while speed of shortening increased 20% for all age groups upon the increase in external calcium from 0.6 mM to 2.4 mM.

DISCUSSION

The major finding of this investigation is that maturation affects the intrinsic contractile state of cardiac muscle, primarily by altering the speed of shortening of the contractile elements, as reflected by *Vs* in isotonic contractions and by force development in isometric contractions. This observation suggests that changes in cardiovascular dynamics in the intact animal during maturation do not result merely from the changes in load placed upon the heart by alteration in metabolism, but rather, it indicates that the changes observed in cardiac index, mean systolic ejection rate, and myocardial fiber shortening rate, result, at least in part, from structural and metabolic alterations associated with maturation. Moreover, these findings suggest that the inotropic properties of the heart are adjusted in a manner appropriate to meet the simultaneously induced alterations in peripheral requirements.

That post-natal development exerts effects on myocardial force development follows from the observation that time to peak tension is prolonged while the rate of tension development is little changed in such a manner that resultant tension is augmented. Muscles from 300-day-old rats develop tension for a longer period of time than 60-day-old rats as shown by a prolonged period of contraction. Thus, development increases the duration of the active state of the myocardium, as measured by the prolongation of the time to peak tension but simultaneously decreases the intensity of the active state as measured by the shift in the force-velocity curve (Fig. 1). These findings suggest that development affects the contractile state of cardiac muscle by varying the rate of interaction at contractile sites, while increasing the number of force developing units.

From these findings, it is apparent that when tension alone is measured, large changes in the contractile state of the myocardium can be overlooked. This fact may account, at least in part, for conflicting data reported in earlier studies of this problem. Studying post-natal development in cats, Davies *et al.* (1975) found an increase in developed tension as a function of age, while Weisfeldt *et al.* (1971b) showed no difference in developed tension between papillary muscles from 6, 12 and 24-month-old male rats. The time to peak tension increases proportionately more than the rate of tension development is abbreviated

(Table 1) and the net effect is an increase in developed force. In addition, Alpert *et al.* (1967) demonstrated a higher calcium activated myosin ATPase activity in 3-month-old rats as compared to 9-month-old rats. This result can be accounted for by the finding of the present study, that as post-natal development proceeds the velocity of muscle shortening decreases.

Therefore, it can be seen that post-natal development profoundly affects the contractile state of cardiac muscle, with force of contraction increasing and speed of shortening decreasing as a function of age. However, the responsiveness of the myocardium to various inotropic agents remains unchanged in the age groups studied.

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Experimental Gerontology, Vol. 17, pp. 205–211, 1982
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AGE-RELATED DIFFERENTIAL EFFECTS OF ZINC ON CONCANAVALIN A-INDUCED CAPPING OF HUMAN LYMPHOCYTES

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(*Received* 15 *September* 1981)

INTRODUCTION

Zinc appears to exert potent biological effects on a variety of cells. Zinc can act as a mitogen when added directly to lymphocyte cultures (Rühl *et al.*, 1971; Berger and Skinner, 1974). Zinc deficiency in animals has been associated with abnormal development of the thymus (Kroneman *et al.*, 1975), the lymphoid system (Tanaka *et al.*, 1978) and defective immune function (Iwata *et al.*, 1979; Fraker *et al.*, 1977; Fernandes *et al.*, 1979). Pharmacological doses of zinc suppress the sickling phenomenon in sickle cell anemia (Arnone and Williams, 1977). The mechanism of action of zinc, however, is not well understood. In the present paper I describe the effects of zinc on concanavalin A (con A)-induced capping in relation to age. The experimental evidence suggests that zinc might act by influencing the microfilament function and that alteration in microfilament function might be an important aspect of the aging phenomenon.

Interaction of ligands with surface receptors of lymphocytes leads to rapid changes in the surface membrane and polarization of the receptors leading to what is termed "cap" formation. Treatment of human peripheral blood lymphocytes (PBL) with fluoresceinated concanavalin A (F-con A) at 37°C induces cap formation in about 10% of the labeled lymphocytes. Cap formation can be altered by cytoskeletal modulating agents such as colchicine (de Petris, 1974; Albertini *et al.*, 1977) and cytochalasin B (CB) (de Petris, 1974; Yahara and Edelman, 1973). It has been proposed that the cell surface receptors are anchored to microtubules and microfilaments and disruption of these elements lead to alterations in surface receptor mobility (Yahara and Edelman, 1973).

Our previous work indicated that zinc modulates lectin-induced mitogenic responses of human PBL in an age-dependent fashion (Rao *et al.*, 1979). Further, we presented evidence indicating that the effect of zinc might be exerted through CB sensitive structures (Rao and Schwartz, 1980). This observation prompted me to study the effect of zinc on con A-induced cap formation of lymphocytes obtained from young and old individuals. The experimental evidence presented in this paper indicates that zinc has a differential ef-

fect on con A-induced lymphocyte capping, analogous to its effect on mitogenic responses (Rao *et al.*, 1979)

MATERIALS AND METHODS

Donors

Blood from young and aged healthy volunteer donors was freshly obtained from a peripheral vein. All donors gave informed consent consistent with the policies of The University of Michigan and the Department of Health and Human Services. The aging population ranged from 65–84 years; the young adult population ranged from 22–35 years.

Isolation of mononuclear cells

Mononuclear cells were prepared from fresh heparinized blood (20^{U}/ml) by the Ficoll-Hypaque density gradient technique. The cells were washed three times with Ca^{++} and Mg^{++} free Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, NY) and resuspended in RPMI-1640 medium (Grand Island Biological Co.).

Drugs

CB and colchicine were obtained from Sigma Chemical Co., St. Louis, MO. Colchicine solution was prepared in water. CB was dissolved in 95% ethanol to a concentration of 2×10^{-3} *M* and appropriate dilutions were made in HBSS. $ZnCl_2$ was obtained from Fisher Scientific Co., NJ. F-Con A was obtained from Calbiochem-Behring Corp., LaJolla, CA.

Capping experiments

Mononuclear cells were suspended at a concentration of 4–6 $\times$ 10^6/ml, and 0.5 ml was used for each experiment. Drugs were added to the cells and the suspension was incubated at 37°C for 30 min with continuous rotation. Then F-con A was added to give a final concentration of 20 μg/ml and incubated for another 30 min. At the end of incubation the cells were fixed with 0.5 ml of 2% paraformaldehyde for 10 min, washed twice with HBSS and the number of caps formed were enumerated under fluorescent microscopy by counting 100 cells. Caps were defined as cells showing less than 1/3 surface fluorescence.

RESULTS

Cap formation with fluoresceinated con A

Fig. 1 illustrates the number of caps formed when the cells were treated with F-con A alone. The number of caps formed varied from 4%–16% and no difference could be detected in results obtained from cells from young and old donors (Student's *t*-test $p = 0.85$).

The monocyte content of our cell suspension did not seem to affect our results since many of the monocytes are lost (perhaps due to adherence to plastic) during the process of incubation at 37°C. Further, monocytes have a tendency to clump and during enumeration of capping under fluorescent microscopy clumped cells were omitted. In one experiment, where monocyte contamination was monitored with latex ingestion, few monocytes were detected at the time of counting, and the results were similar to non-latex ingested specimens. Thus, although a mononuclear cell preparation was used throughout these experiments the differences noted could be attributed mainly to lymphocytes.

Effect of colchicine on con A-induced capping

Preincubation with colchicine (10^{-5} *M*, final concentration) increased the number of

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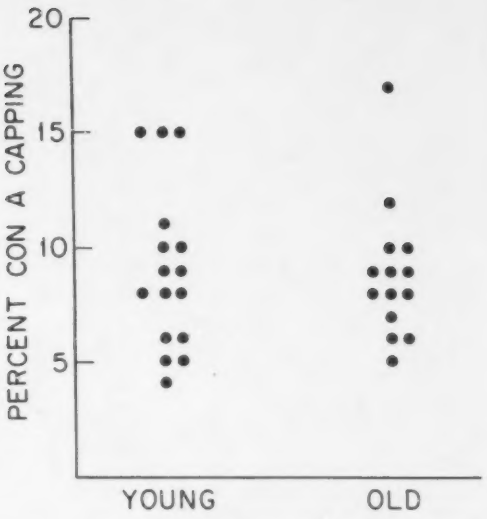

FIG. 1. Con A-induced capping of human peripheral blood lymphocytes. Lymphocytes were treated with 20 μg/ml (final concentration) of F-Con A and the number of caps formed were enumerated under fluorescent microscopy. Age of the young individuals was between 22–35 years and old individuals 65–84 years. In this and all subsequent figures each point represents data derived from a different cell donor.

caps formed in both groups (Fig. 2). The number of caps formed in the young population was 47 ± 15 (mean ± 1 SD), whereas in the old group it was 57 ± 9 (mean ± 1 SD). Statistical analysis by the Mann-Whitney used statistic test gave a value of 62.5 which was significant at $p = 0.024$.

Effect of colchicine plus zinc on con A-induced capping

Preincubation with zinc alone, followed by F-con A treatment failed to bring about a significant change in the number of caps formed as compared to treatment with F-con A alone (F-con A − 6%, zinc and F-con A − 5%). However, when mononuclear cells were incubated with both colchicine (10^{-5} *M*) and zinc (10^{-4} *M*) together for 30 min followed by F-con A treatment and compared with colchicine pre-treatment alone, dramatic difference was observed in the number of caps formed between the two groups (Fig. 3). Addition of zinc caused further enhancement of the number of caps formed in the younger age group as compared to the effect of colchicine alone, whereas in the aged population the number of caps decreased when compared to colchicine alone. The difference was highly significant ($p = 0.0002$) using the Wilcoxan Rank-Sum test.

Effect of colchicine plus cytochalasin B on con A-induced capping

The effect of CB on con A-induced capping was studied at two concentrations 10^{-6} *M* and 10^{-7} *M* (Fig. 4 a,b). At a concentration 10^{-6} *M* of CB (Fig. 4a) the number of caps formed decreased in both groups as compared to pretreatment with colchicine alone. At

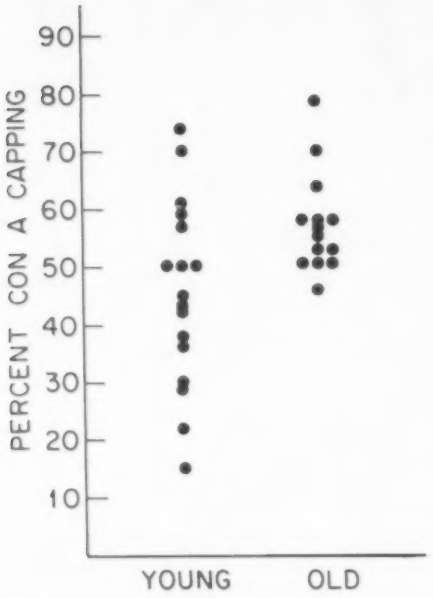

FIG. 2. Effect of colchicine on Con A-induced capping. Lymphocytes were pretreated with colchicine (10^{-5} *M*, final concentration) for 30 min and then incubated with F-con A for an additional 30 min.

10^{-7} *M* the effect was variable in both groups (Fig. 4b). Statistical analysis revealed no difference between the groups at these two concentrations of CB.

DISCUSSION

The experimental results show that treatment of human PBL with con A alone does not show any difference in the capping phenomenon between the young and aged population. This is in contrast to a recent report in which a difference in capping was noted between young and old individuals using con A alone (Naeim and Walford, 1980). This discrepancy could be due to the difference in the age of the older group which ranged from 80 to 98 in their study and 65 to 84 in my study. However, use of colchicine and zinc elicited significant differences between the young and old population in the present evaluation.

I offer the following interpretation for the observations described in this paper. It has been proposed that cell surface receptors are anchored to microtubules and microfilaments, and these structures have been attributed interrelated but opposite roles in maintaining the distribution and the mobility of surface receptors (Poste *et al.*, 1975 a,b). The inability of con A alone to demonstrate any difference between the two groups studied may be attributed to the anchoring effect of microtubules which might mask the differences in microfilament function. Once the microtubules are disrupted by colchicine, microfilaments, now unopposed, can bring about enhancement in capping and whatever differences exist between the microfilaments of lymphocytes from young and old persons become apparent. In these experiments when colchicine alone was used the differences

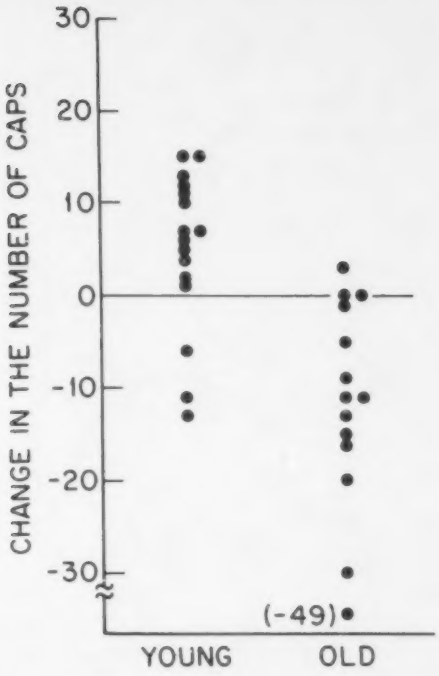

FIG. 3. Effect of colchicine plus zinc on con A-induced capping. Lymphocytes were pretreated with colchicine (10^{-5} *M*, final concentration) plus zinc (10^{-4} *M*, final concentration) for 30 min and then incubated with F-con A for an additional 30 min. The caps formed with colchicine plus zinc treatment were compared to colchicine treatment alone and the difference in the number of caps, per 100 cells counted, was plotted.

were significant at $p = 0.024$. But when zinc was added in addition to colchicine the difference was even more dramatic ($p = 0.0002$). Our previous observation that zinc modulates mitogenic responses by affecting CB sensitive structures (Rao and Schwartz, 1980) is consistent with the present finding that zinc is involved in modulating the capping phenomenon—another microfilament dependent function.

CB completely inhibits capping at high concentrations. For this reason the effect of CB on capping was tested at very low concentrations of CB; unlike zinc, CB does not show any age-dependent effects. This suggests that although both these agents act on microfilaments they might exert their action at different sites among the steps involved in microfilament formation or function. CB has been shown to inhibit actin polymerization by blocking filament elongation (Lin *et al.*, 1980). It is conceivable that zinc might be acting on some other molecule which might also have a role in microfilament formation or function. Calmodulin, a calcium regulating protein, is found in close association with microfilaments (Welsh *et al.*, 1978). It was proposed that the effect of zinc on cellular function might be mediated through its inhibitory action on calmodulin (Brewer *et al.*, 1979). Trifluoperazine, a known inhibitor of calmodulin (Levin and Weiss, 1977), can also in-

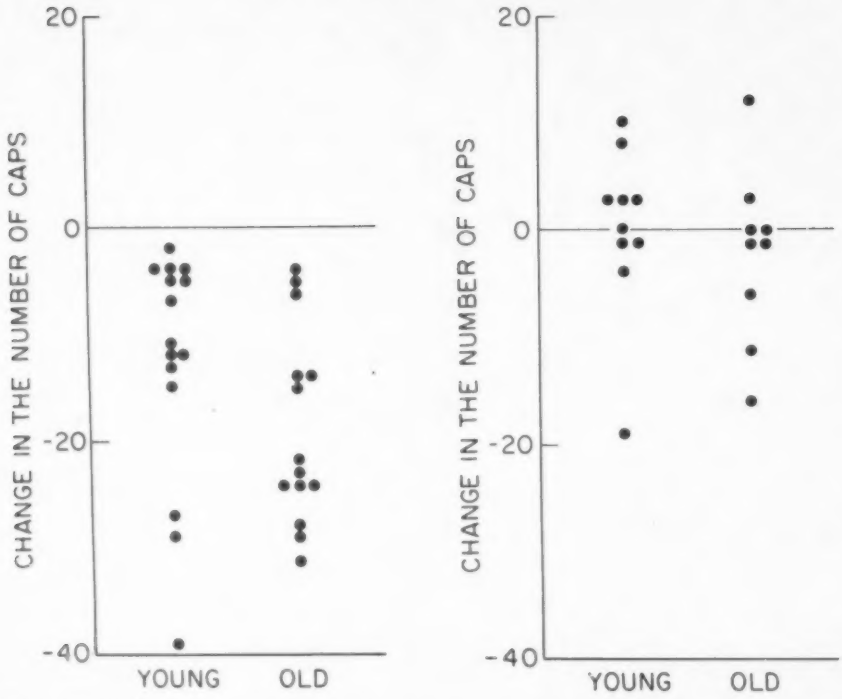

FIG. 4. Effect of colchicine plus cytochalasin B on con A-induced capping. Lymphocytes were preincubated with colchicine (10^{-5} *M*, final concentration) plus cytochalasin B (10^{-6} *M*, Fig. 4a; 10^{-7} *M*, Fig. 4b). Experimental protocol was similar to that described in Fig. 3.

fluence con A-induced capping (Bourguignon and Balazovich, 1980). Thus, the observations reported in this paper might be pointing to an important area governing cell function which might be relevant to the study of the aging process. Experimental approaches to the study of actin polymerization and calmodulin function in relation to age might yield fruitful results in our quest to understand the molecular mechanism of senescence.

SUMMARY

Con A-induced capping of lymphocytes is altered by a number of cytoskeletal modulating agents. Zinc, in the presence of colchicine, demonstrated an age-dependent differential effect on con A-induced capping of human peripheral blood lymphocytes. In general, zinc enhanced capping of lymphocytes from young individuals and suppressed from those of old individuals.

The effect of zinc on the cap formation adds further support to the evidence that zinc might be acting on the microfilaments. However, cytochalasin B, another microfilament modulating agent, does not show a differential effect similar to zinc. This suggests that zinc might modify microfilament formation or function in a manner different from cytochalasin B. The proposal that the effects of zinc might be due to its action on calmodulin suggests that calmodulin, the calcium regulating protein, may have a role in the physio-

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logical processes associated with aging. Further studies in this area might yield fruitful results in our understanding of the molecular mechanism of senescence.

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Experimental Gerontology, Vol. 17, pp. 213–217, 1982
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QUERCETIN, FLAVONOIDS AND THE LIFE-SPAN OF MICE

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(*Received* 15 *September* 1981)

Abstract—A dietary supplement of 0.1% quercetin significantly reduced the life span of mice. The effect was predominantly on the 'shorter living' males. A blackcurrant juice extract, containing a mixture of flavonoids in addition to quercetin, prolonged significantly the life span of the 'older dying' females. The significance of these results *vis-a-vis* aging mechanisms and the dietary intake of quercetin is discussed.

INTRODUCTION

QUERCETIN (3,5,7,3′,4′-pentahydroxyflavone) and its glycosides, such as rutin (quercetin-3-rutinoside), are of widespread occurrence in plants (Herrmann, 1976; Kühnau, 1976). Little, however, is known of their metabolic significance in man despite their not inconsiderable intake in some diets.

There is evidence that cholesterol metabolism in experimental animals is influenced by dietary quercetin (Basarker and Hatwalne, 1975; Jones and Hughes, unpublished data) and it is well established that in guinea pigs the concentration of tissue ascorbic acid may be modified by dietary flavonoids (Hughes and Wilson, 1977).

Of possibly greater significance are recent indications that quercetin, in *in vitro* tests, possesses considerable direct mutagenicity (Bjeldanes and Chang, 1977; Kimura *et al.*, 1979; Sugimura, 1979)—a finding which could carry important implications in terms of human nutrition. The experiment described in this report was designed to examine the influence of quercetin and other dietary flavonoids on the life-span of mice.

Three groups (A,B,C) of 5-week-old mice (strain LACA, Medical Research Council) were used. Each group contained 50 males and 50 females. Group A received a standard semi-synthetic diet MG1, originally designed as a scorbutogenic diet and shown to be virtually flavonoid-free (Williams and Hughes, 1972). Group B received MG1 to which 'single strength' blackcurrant juice concentrate had been added at the pre-baking stage (220 ml juice per 1000 g diet). The blackcurrant juice concentrate was used as a source of mixed flavonoids; it provided, per 1000 g diet, an estimated 380 mg of total flavonoid material of which c. 40 mg were present as quercetin and its glycosides and the bulk of the remainder as anthocyanins. Group C received MG1 to which 0.1% quercetin had been added; this provided an estimated daily intake of c. 10 mg quercetin per animal.

Citric acid (a high dietary intake of which had been previously shown [Wright and Hughes, 1976] to influence the life span of mice) and ascorbic acid were added to diets A

TABLE 1. INFLUENCE OF BLACKCURRANT CONCENTRATE (GROUP B) AND QUERCETIN (GROUP C) ON CHANGE IN BODY WEIGHT OF MICE. (MEAN WEIGHTS WITH THEIR STANDARD ERRORS.)

Group	Sex	Time (Weeks) 6	12	24	50
A (control)	male	22.3 ± 0.3	30.9 ± 0.8	34.6 ± 0.9	34.5 ± 0.7
	female	20.2 ± 0.4	26.2 ± 0.7	29.4 ± 0.9	29.3 ± 0.5
B (blackcurrant)	male	21.9 ± 0.1	30.2 ± 0.2	33.2 ± 0.2	34.3 ± 0.5
	female	21.1 ± 0.2	26.8 ± 0.4	29.9 ± 0.4	30.1 ± 0.4
C (quercetin)	male	23.1 ± 0.2	31.4 ± 0.4	35.1 ± 0.6	36.2 ± 0.7
	female	20.6 ± 0.1	26.2 ± 0.2	29.0 ± 0.3	29.3 ± 0.3

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and B in quantities equivalent to those introduced into C by the blackcurrant juice concentrate.

RESULTS

The results are summarized in Tables 1 and 2 and in Fig. 1 and 2. Food intake measurements revealed no differences between the groups, nor were there any significant differences between the times taken to attain a stable mature body weight (Table 1).

The main finding to emerge from the study was a significant overall reduction in the life-span of the quercetin-supplemented group (Kruskall-Wallis rank test [Hull and Nie, 1979]; Fig. 1, Table 2). Analysis of the results revealed that the 'quercetin effect' was attributable to the males rather than the females and that it influenced the 'shorter life-span' animals rather than the 'longer life-span' ones. The life span of the 15 'last dying' animals was unaltered by the quercetin.

The blackcurrant juice concentrate, on the other hand, had no significant influence on the mean overall life span but prolonged significantly the life span of the 'last 15' females (Table 2) (Student's *t*-Test).

DISCUSSION

The effect of quercetin in shortening the life span of mice is perhaps counter to what one would expect on theoretical grounds. Quercetin is a potent antioxidant (Kühnau,

TABLE 2. INFLUENCE OF BLACKCURRANT CONCENTRATE (GROUP B) AND QUERCETIN (GROUP C) ON MEAN LIFE-SPAN (WEEKS) OF MALE AND FEMALE MICE. MEAN LIFE-SPANS WITH THEIR STANDARD ERRORS.

Group	A (control)	B (blackcurrant)	C (quercetin)
Mean for group (males & females)	111.3 ± 2.0	113.5 ± 2.5	100.3 ± 2.6*
Females (total)	116.4 ± 2.8	120.7 ± 3.4	109.6 ± 3.5
Females (first 15)	90.5 ± 3.3	87.5 ± 4.4	80.9 ± 4.3
Females (last 15)	136.8 ± 0.6	141.7 ± 0.8*	138.5 ± 1.7
Males (total)	106.3 ± 2.8	106.9 ± 3.4	91.3 ± 3.3*
Males (first 15)	79.7 ± 2.8	75.1 ± 3.8	65.9 ± 2.1*
Males (last 15)	121.7 ± 7.9	130.9 ± 0.9	122.4 ± 3.1

*Difference between group mean and control mean significant. $P < 0.01$.

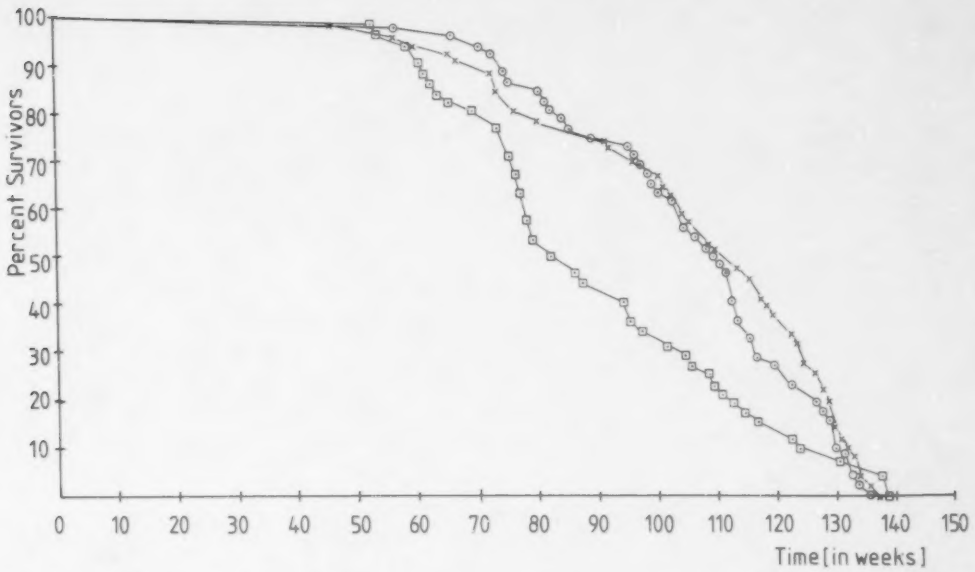

FIG. 1. Survival curves for male mice given dietary supplements of quercetin (⊡—⊡—⊡) or blackcurrant juice (–x–x–) compared with control mice (⊖—⊖—⊖).

1976) and there is evidence that antioxidants, presumably because of a free-radical scavenging action, may increase the life span of experimental animals (e.g. Comfort *et al.*, 1971; Miquel and Economos, 1979). Again, quercetin stabilizes ascorbic acid, retarding its chemical degradation (Hughes and Wilson, 1977). There are indications that breakdown products of ascorbic acid are mutagenic; quercetin, by reducing the formation of such products, would perhaps retard the aging process(es) (Burnet, 1974; Hughes, 1981).

The results of our experiment would appear to suggest that any such general advantages to be expected from flavonoid supplementation are, in the case of quercetin, obscured by less advantageous attributes. Of significance in this respect are reports of the comparatively high mutagenicity of quercetin when tested by *in vitro* techniques (Bjeldanes and Chang, 1977; Kimura *et al.*, 1979; Sugimura, 1979); a quercetin-induced reduction of life-span would therefore be consistent with a theory of aging of the somatic mutation type.

Tissue deprivation of trace elements could also be a contributory factor of significance. Quercetin is able to form chelation compounds with certain metals such as copper (Kühnau, 1976). Prolonged ingestion of quercetin could conceivably result in a state of chronic deficiency of one or more of the essential elements which would, in turn, be reflected as a reduction in life-span. A similar mechanism has been proposed to account for the reduced life-span of mice receiving elevated dietary intakes of citric acid (Wright and Hughes, 1976).

The indication that the blackcurrant extract prolonged the life-span suggests a different mode of action and is more in agreement with the type of result to be expected on a theoretical basis. Quercetin is a relatively minor component of the blackcurrant juice flavonoid complex (Morton, 1968) and it would appear that its deleterious activity is masked by the 'beneficial' influence of the other flavonoids present. The clear difference

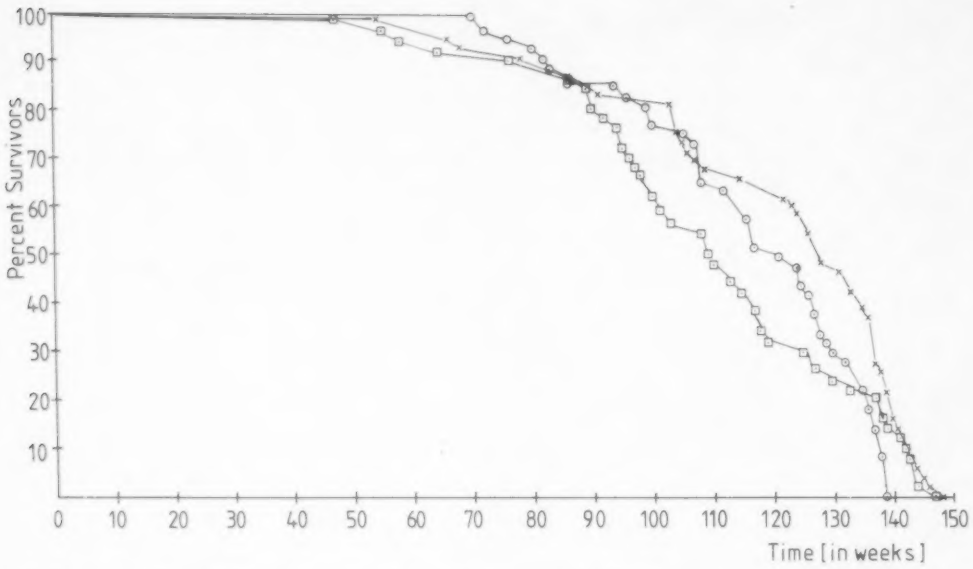

FIG. 2. Survival curves for female mice given dietary supplements of quercetin (⊡——⊡) or blackcurrant juice (–x–x–) compared with control mice (⊖——⊖——⊖).

between the effects of the two dietary supplements underlines the need for caution in generalizing about 'life-prolonging' effects of members of the same group of dietary compounds.

Of greater interest, perhaps, is the result of the analysis of deaths in terms of 'early dying' and 'late dying' mice. The quercetin supplement modified, primarily, the deaths of the 'early dying' animals whereas the 'blackcurrant flavonoid' effect appeared to be located in the 'later dying' animals, thus indicating a *qualitative* difference between the mode of action of the two supplements. In other words, one should perhaps distinguish between a chronic toxicity, likely to influence preferentially animals that, for constitutional reasons are in any case likely to die young, and any 'true' life-span effect which would be located predominantly in those animals genetically predisposed to longevity. Clarke and Maynard-Smith made essentially the same point in seeking to distinguish between ageing *per se* and a reduced 'threshold of vitality' (Clarke and Maynard-Smith, 1961). Analysis of life-span studies in terms of 'early dying' and 'late dying' groups could perhaps serve to remind us that 'prolongation of living' is not necessarily to be qualitatively equated with 'prevention of death.'

The human implications of this study are not without interest. Quercetin is widely distributed in the plant kingdom, typical values being (mg/100g fresh-weight): bilberry 5.0–15.0; blackcurrant 4.0–8.0, cherry 2.0–8.0, chives 30.0, and lettuce 2.0–25.0 (Herrmann, 1976). Usually, however, the quercetin is concentrated in the outer, non-edible layers of fruit and vegetables, the more frequently eaten fleshy parts being virtually quercetin-free. Asparagus tips contain less than 1.0 mg/100 g whereas the leaves contain c. 400 mg/100 g. Onions are of particular interest as the outer dry scales may contain up to 6.5%

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quercetin whereas the inner portions of the bulb are comparatively quercetin-free (Herrmann, 1976; Eleri Jones, R.J. Hurley and R.E. Hughes, unpublished data).

The *per capita* daily intake of quercetin in the average diet is therefore unlikely to exceed some 50 mg. On the other hand, salad fiends, those who eat their tomatoes, apples and onions unpeeled, and 'natural food' addicts who indiscriminately include a range of esoteric plant material in their dietaries, could well ingest some 200–500 mg of quercetin daily.

Acknowledgements—Eleri Jones was in receipt of a Beecham Products Research Studentship. Mr Michael Ford (Beecham Products, Royal Forest Factory, Coleford) supplied the blackcurrant juice concentrate.

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Experimental Gerontology, Vol. 17, pp. 219–225, 1982
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MONOAMINE OXIDASE ACTIVITIES IN LIVER, HEART, SPLEEN AND KIDNEY OF THE RAT

Organ-Specific Changes in Aging and After Chronic Manganese Chloride Administration

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(*Received* 15 *September* 1981)

INTRODUCTION

THERE IS increasing evidence that monoamine oxidase (MAO; Monoamine: oxygen oxidoreductase [deaminating], EC 1.4.3.4), a key enzyme responsible for the metabolism of biogenic amines in various tissues, may exist in more than one form (Sandler and Youdim, 1972; Tipton *et al.*, 1976). Based on substrate and inhibitor specificities, the classification into two forms, A and B, has been widely accepted (Johnston, 1968; Sandler and Youdim, 1972; Tipton *et al.*, 1976). Serotonin is an A-type substrate (Johnston, 1968) whereas benzylamine (Tipton *et al.*, 1976) and phenylethylamine (Suzuki *et al.*, 1979; Lewinsohn *et al.*, 1980; Peers *et al.*, 1980; Kinemuchi *et al.*, 1980) are B-type substrates. Clorgyline (Johnston, 1968) and deprenyl (Knoll and Magyar, 1972) are respectively selective A-type and B-type inhibitors.

The aims of the present study were:

(A) To investigate the possibility that in aging there may be differential changes of type A and type B MAO activities in various organs (i.e. follow a pattern that is unique to a particular organ) as suggested by three types of observations. Firstly, in the brain of the human (Robinson *et al.*, 1972; Shih, 1979; Fowler *et al.*, 1980) and the rat (Benedetti and Keane, 1980; Lai *et al.*, 1981a; Leung *et al.*, 1981a) the age-related alterations in type A MAO activities differ from those in type B MAO activities. Secondly, sex-steroids appear to affect these two types of MAO activities differentially depending on the target organ itself (McEwen *et al.*, 1978; Leung *et al.*, 1980). Thirdly, it is well recognized that in mam-

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malian aging certain hormonal disturbances do occur (see Finch, 1976, for discussion); the disappearance of ovarian cycles and the consequent large decrease in ovarian output of sex-steroids are phenomena especially relevant.

(B) To determine if chronic and life-long treatment with manganese chloride exerted any differential effects on the age-related changes (if any) in type A and type B MAO activities in organs other than the brain since it has already been demonstrated that such a treatment modulates the age-related changes in these activities in various rat brain regions (Lai *et al.*, 1981a; Leung *et al.*, 1981a).

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MATERIALS AND METHODS

Animals

Wistar (M.R.C. Porton Strain) rats were used in all the experiments. The developmental rat model of chronic manganese toxicity was set up as described previously (Lai *et al.*, 1981b,c). In brief, rats were exposed to manganese chloride (1 mg of $MnCl_2 \cdot 4H_2O$ per ml of drinking water) from conception. The animals were exposed to the metal before weaning via their mother's circulation and milk; at weaning and subsequently they were directly exposed to the metal in drinking water. Treatment of some of the animals was continued for over 2 years. The body weights and water intake rates of manganese-treated rats were not significantly different from those of age- and sex-matched control animals. In the present study control and manganese-treated young (2-month-old) and control and magnanese-treated old (24-to-28-month-old) female rats were employed. In each experiment at least two animals from each group were used.

Preparation of tissue homogenate

Rats were decapitated and the organs (liver, heart, kidneys and spleen) were rapidly dissected out. The heart was cut open so that any residual blood could be removed by blotting with tissue paper as was blood on other organs. Each organ was frozen on dry ice and kept at $-20°C$ overnight. On the following day tissue homogenates were prepared as previously described (Lai *et al.*, 1980). (Preliminary experiments indicated that storage of these organs in this manner did not result in any loss of MAO activities). The homogenate of liver was prepared as described by Lai *et al.*, (1980) and other tissues essentially by the procedure described for brain tissue (Lai and Clark, 1979) except for a different isolation medium (Lai *et al.*, 1980).

Assay of monoamine oxidase activities

This was by the method of Owen *et al.*, (1977) with minor modifications (Leung *et al.*, 1980, 1981a). Enzymic activities were determined with serotonin (type A substrate) and benzylamine (type B substrate) at final substrate concentrations of 1 mM (Leung *et al.*, 1980, 1981a). Under these assay conditions, enzymic activities showed linear increases with respect to increasing incubation time and increasing amounts of tissue.

Protein determination

This was carried out by the method of Lowry *et al.*, (1951).

Statistical analysis of results

This was performed using the non-paired two-tailed *t*-test and only P values < 0.05 were regarded as significant.

RESULTS

Effect of chronic treatment with manganese chloride on monoamine oxidase activities in liver, heart, spleen and kidney of the rat

Chronic treatment with manganese chloride from conception did not alter the relative distribution of serotonin-oxidizing (MAO-A) and benzlamine-oxidizing (MAO-B) ac-

tivities in these organs in young and old animals, comparisons being made with the appropriate age- and sex-matched control animals (Tables 1 and 3). However, manganese treatment did result in a small (12%) but significant ($P < 0.05$) increase in hepatic serotonin-oxidizing activities in young rats (Tables 1 and 2).

Manganese treatment resulted in small decreases in the MAO type A/B ratio in spleen and kidney in younger rats and in all four organs in old animals (Table 3).

Effect of aging on monoamine oxidase activities in liver, heart, spleen and kidney of the rat

Small but significant increases in hepatic MAO-A (21%; $P < 0.001$) MAO-B (18%; $P < 0.05$) activities were found in control rats in aging (Tables 1 and 2). In manganese-treated animals, the slight age-related increases in hepatic MAO-A and MAO-B activities were not statistically significant ($P > 0.05$; Tables 1 and 2) although both activities in manganese-treated aged rats were significantly higher (17–26%; $P < 0.02$) than in control young rats (Tables 1 and 2). However, in manganese-treated animals only, there was an age-related decrease in the hepatic MAO A/B ratio (Table 3).

In control rats in aging there were substantial increases in cardiac MAO-A (127%; $P < 0.001$) and MAO-B (200%; $P < 0.001$) activities (Tables 1 and 2). Similarly, there

TABLE 1. EFFECTS OF AGING AND OF CHRONIC MANGANESE CHLORIDE TREATMENT ON MONOAMINE OXIDASE ACTIVITIES IN RAT ORGANS.

Monoamine oxidase activities*		Rats	Liver	Heart	Spleen	Kidney
Serotonin-oxidizing (Type A)	Young	Control (6)	2.56 ±0.21	1.01 ±0.18	0.19 ±0.03	0.57 ±0.03
		Manganese-treated (4)	2.87 ±0.13	0.86 ±0.21	0.20 ±0.02	0.54 ±0.03
	Old	Control (6)	3.09 ±0.10	2.29 ±0.37	0.19 ±0.05	0.49 ±0.07
		Manganese-treated (4)	3.00 ±0.10	2.50 ±0.30	0.16 ±0.02	0.50 ±0.03
Benzylamine-oxidizing (Type B)	Young	Control (6)	1.42 ±0.22	0.08 ±0.02	0.04 ±0.01	0.13 ±0.02
		Manganese-treated (4)	1.55 ±0.29	0.07 ±0.01	0.05 ±0.01	0.14 ±0.01
	Old	Control (6)	1.68 ±0.21	0.24 ±0.06	0.05 ±0.02	0.10 ±0.01
		Manganese-treated (4)	1.79 ±0.14	0.28 ±0.06	0.05 ±0.01	0.11 ±0.01

*Enzymic activities were expressed in nmol of product formed per minute per mg of protein; all values were means ± SDs with the number of experiments in parentheses.

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TABLE 2. LEVELS OF STATISTICAL SIGNIFICANCE OF THE COMPARISON OF MONOAMINE OXIDASE ACTIVITIES BETWEEN THE GROUPS BY THE TWO-TAILED *t*-TEST.

Comparison between groups	Monoamine oxidase activities (type)	Liver	Heart	Spleen	Kidney
Manganese-treated young versus Control young	A	0.05			
	B				
Control young versus Control old	A	0.001	0.001		0.05
	B	0.05	0.001		0.01
Manganese-treated young versus Manganese-treated old	A		0.001	0.05	
	B		0.001		0.005
Manganese-treated old versus Control young	A	0.005	0.001		0.01
	B	0.02	0.001		
Control old versus Manganese-treated young	A	0.02	0.001		
	B		0.001		0.001

The enzymic activities shown in Table 1 were analyzed statistically. Only *P* values less than 0.05 were quoted as being significant.

were age-related increases in cardiac enzymic activities in manganese-treated animals, but which were greater (191% increase in MAO-A and 300% increase in MAO-B activities; $P < 0.001$) than those seen in control animals (Tables 1 and 2). In control as well as in manganese-treated rats, a greater age-related increase in cardiac MAO-B activities than in MAO-A activities (Table 1) was reflected in an age-related decrease in the MAO-A/B ratio in this tissue (Tables 1 and 3).

Small but significant decreases in renal MAO-A (14%; $P < 0.05$) and MAO-B (23%; $P < 0.01$) activities were observed in control rats in aging (Tables 1 and 2). In manganese-

TABLE 3. SEROTONIN-OXIDIZING TO BENZYLAMINE-OXIDIZING ACTIVITY RATIO (MAO A/B RATIO) IN VARIOUS RAT ORGANS: EFFECTS OF AGING AND OF CHRONIC MANGANESE CHLORIDE TREATMENT.

Rats		MAO A/B Ratio*			
		Liver	Heart	Spleen	Kidney
Young	Control	1.80	12.63	4.75	4.39
	Manganese-treated	1.85	12.29	4.00	3.86
Old	Control	1.84	9.54	3.80	4.90
	Manganese-treated	1.68	8.93	3.20	4.55

*All values are the means of four to six experiments.

treated animals, the only significant age-related decrease was in MAO-B activities (21%; $P < 0.005$; Tables 1 and 2). In manganese-treated and control rats, the MAO A/B ratio was decreased in this tissue in aging (Table 3).

Although the only age-related change in the spleen was a small (20%; Table 1) but significant ($P < 0.05$; Table 2) decrease in MAO-A activity in manganese-treated rats, but not control rats, there was an age-related decline in the MAO A/B ratio in this tissue in both control and manganese-treated animals (Table 3).

DISCUSSION

Our results demonstrate that aging exerts organ-specific effects on type A and type B MAO activities in the various organs of the rat (Tables 1 and 3). These effects can be separated into three broad categories: (1) an increase in both activities, as in the heart and liver; (2) a decrease in both, as in the kidney; and (3) no significant changes in both, as in the spleen. These changes seen in aging are very different from those seen during development (Leung *et al.*, 1981b) as exemplified by the changes in the MAO A/B ratio (Table 3). Thus, whereas the MAO A/B ratio in both the heart and the liver shows definite age-dependent increases during development (Leung *et al.*, 1981b), it was unchanged in the liver and decreased in the heart in aging. The MAO A/B ratio, after an initial decrease, remains constant in the spleen during development (Leung *et al.*, 1981b) but is decreased in aging. Furthermore, the MAO A/B ratio in the kidney shows age-dependent decreases during development (Leung *et al.*, 1981b) but is slightly increased in aging. Hence the changes seen in aging are not an extension of age-related developmental changes.

Chronic treatment of rats with manganese chloride from conception results in increases in the metal concentration which vary from organ to organ (Chan *et al.*, 1981; Lai *et al.*, 1981b; Lai *et al.*, 1981c). Consequently, it is not surprising that the effects of manganese treatment on the age-related changes in MAO activities are different in the various organs (Tables 1–3; also see Lai *et al.*, 1981a; Leung *et al.*, 1981a). In the heart the decrease in the MAO A/B ratio during aging is more pronounced in manganese-treated than in control rats (Table 3) although the cardiac manganese concentration in the treated rats is only slightly (and insignificantly) higher (Chan *et al.*, 1981). The same manganese treatment significantly raises the manganese concentration in the liver, kidney and spleen (Chan *et al.*, 1981) which all display a decrease in the MAO A/B ratio with aging, not exhibited by control rats (Table 3). The changes in aging in both the kidney and the spleen in manganese-treated rats also do not parallel those in control animals (Table 3). These results suggest that chronic treatment with manganese exerts modulatory effects on the organ-specific aging changes in MAO activities (see also Lai *et al.*, 1981a; Leung *et al.*, 1981a).

Since MAO activities are dependent on the microenvironment of the membrane, changes in the membrane composition—particularly of phospholipids—during aging could lead to altered functional expression of MAO. Huang and Faulkner (1980) have studied the effects of membrane perturbation (using phospholipases and lipid-substitution) on the functional expressions of type A and B brain mitochondrial MAO activities *in situ*. Such treatments decrease the membrane content of phophatidyl choline (90%), sphingomyelin (50%), and phosphatidyl ethanolamine (30%) which in turn increases the fluidity of the hydrophobic core of the membrane. The resultant increased membrane fluidity leads to a specific inactivation (25%) of type A but not B MAO activity. Conversely, when liposomes made of phosphatidyl choline, phosphatidyl ethanolamine, cardiolipin or phosphatidic acid (Huang and Faulkner, 1980) are incubated with brain mitochondria, type B MAO ac-

tivity is decreased. This suggests that surface charge and polar interactions preferentially inhibit type B MAO activity (Huang and Faulkner, 1980). Since in rat heart mitochondria the contents of negatively charged lipids—particularly cardiolipin, phosphatidic acid, phosphatidylserine and phosphatidyl-glycerol—are decreased during aging (Nohl and Kramer, 1980) this should enhance cardiac type B MAO activity. The observed increase in cardiac type B MAO activity during aging (Table 1 and 2) is consistent with such a prediction. Nohl and Kramer (1980) also observed that the phospholipid complement of polyunsaturated fatty acids (except arachidonic) and of saturated fatty acids are respectively decreased and increased and that the unsaturated to saturated membrane lipid ratio in rat heart mitochondria is decreased (Nohl and Kramer, 1980) during aging. These changes are also accompanied by a distinct *decrease* in lipid fluidity of the bulk phase of membrane lipids (Nohl and Kramer, 1980). These changes would enhance type A MAO activity since an *increase* in membrane fluidity selectively inactivates it (Huang and Faulkner, 1980). This prediction is again fulfilled by an observed increase in type A activity in the heart during aging (Table 1).

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It is also well established that ovarian aging results in cessation of the estrous cycle and greatly decreased sex-steroid secretion (Finch, 1976). Sex-steroids exert receptor-mediated (McEwen *et al.*, 1978; White *et al.*, 1978; Marr *et al.*, 1980) and organ-specific (Leung *et al.*, 1980) effects in a variety of target organs, which may include the kidney (see Fanestil and Park, 1981 for a review of evidence). Some of the age-related changes in MAO activities in the organs (Tables 1–3) could therefore have originated, at least in part, from altered regulation as a result of decreased sex-steroid secretion. Our conclusions are that the organ-specific and differential effects on type A and B MAO activities in various rat organs in ageing may be attributable to: (1) changes in membrane fluidity; (2) age-related alterations in the regulation by sex-steroids; or (3) a combination of (1) and (2). These should prove to be invaluable subjects for further investigations on the mechanisms that underlie enzymatic changes in aging.

SUMMARY

The effects of aging and chronic manganese chloride administration on monoamine oxidase (MAO, EC 1.4.3.4.) activities in liver, heart, spleen and kidney were studied in 2-month-old and 24-28-month-old rats. In control rats in aging, serotonin-oxidizing (type A) and benzylamine-oxidizing (type B) activities were slightly increased in the liver, markedly increased in the heart, decreased in the kidney, and unchanged in the spleen. Chronic manganese treatment from conception for over 2 years enhanced or abolished the age-related increases in both types of activities in the heart and the liver respectively, abolished the age-related decrease in type A activity in the kidney and decreased type A activity in the spleen. In the manganese-treated animals, the age-related decreases in MAO A/B ratio in the heart and the spleen and increase in the kidney were more pronounced than corresponding changes in control animals. Furthermore, there was an age-dependent decrease in the MAO A/B ratio in the liver not observed in control rats. In the younger rats the only detectable effect of chronic manganese treatment was a small increase in type A activity in the liver. These results suggest that: (1) aging exerts organ-specific and differential effects on MAO heterogeneity in various rat organs, and (2) chronic manganese treatment modulate these age-related effects. These findings are briefly discussed in relation to: (1) changes in development, (2) changes in the structure and function of

mitochondrial membrane lipids in aging, (3) organ distribution of manganese in chronic manganese toxicity, and (4) decreased secretion of sex-steroids in aging.

Acknowledgments—We thank Professor A.N. Davison for his encouragement and interest in our work. The generous support of the Worshipful Company of Pewterers and the Brain Research Trust is gratefully acknowledged.

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Experimental Gerontology, Vol. 17, pp. 227–233, 1982
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EFFECT OF PROTEIN MALNUTRITION ON THE CROSSLINKING OF DERMAL COLLAGEN IN AGING

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(*Received* 15 *September* 1981)

INTRODUCTION

IT HAS long been known that nutrition has a marked influence on the survival of an organism during the growth period. During the process of aging, changes in the collagen increase its aggregation and crosslinks (Joseph and Bose, 1960). The tensile strength, shrinkage temperature and isometric tension were found to increase on aging due to an increase in the crosslinking (Brown and Consden, 1958; Verzar, 1964; Vogel, 1978). It was reported that the digestibility of amorphous collagen decreased with age (Hamlin *et al.*, 1975, 1978). The hexosamine/hydroxyproline ratio in the skin also decreased with age (Ohori *et al.*, 1973). It was observed that the synthesis and content of neutral salt-soluble collagen and the excretion of urinary hydroxyproline decreased in protein malnutrition (Alleyne *et al.*, 1969; Rao and Bose, 1969; Anasuya and Rao, 1970; Hill *et al.*, 1970; McClain *et al.*, 1975; Mangonidi *et al.*, 1979; Rao and Rao, 1980a, 1980b; Tanaka *et al.*, 1980). The present study has been undertaken to learn the effect of protein deficiency on crossiinking with aging.

MATERIALS AND METHODS

A sufficient number of 3-week-old female albino rats of the same breeding colony (King Institute, Madras) were used throughout the experiment. The animals were divided into three batches A, B and C. The animals of Batch A (3 weeks old) were fed the basal diet for 1 week and then divided into two groups. The animals of batch B and C were allowed to continue with commercial rat feed (Hindusthan Lever, Bombay). When the animals of Batch B attained 10 mo of age they were fed the basal diet for 1 week and then divided into two groups. Similarly when the animals of batch C were 20 mo old, they were divided into two groups and fed the basal diet for 1 week. In each case the first group was allowed to continue with the basal diet (20% casein) as it was kept as control and the other group was fed a low protein diet (6% casein). In each batch, animals in the two groups—control and protein-deficient—were pair fed. Water was freely available. Daily records for food consumption were kept and body weights were taken every week. On the 28th day, the rats were killed by decapitation and the skins were removed.

For the convenience of analysis, six skins in each group were pooled together to make one sample, thus making six samples in each group. The skins were depilated with a razor blade, fleshed and washed for analysis.

Reversibility of neutral salt-soluble collagen gel

The pooled samples of skins were finely minced and the neutral salt-soluble collagen was extracted according to Levene and Gross (1959) using 1.0 M NaCl. An aliquot of the extract from each pooled sample was purified by the method of Piez *et al.*, (1963) and the percent of reversibility of the collagen gel was determined according to the method of Hausmann (1963). Extracts of 0.04% collagen concentration in phosphate buffer (pH 7.4) were gelatinized for 24 h at 37°C and then kept at 4°C for 24 h. The gel formation was determined by measuring optical density (O.D.) at 640 nm and 37°C and again at 4°C. The percentage reversibility of a gel was calculated by the formula:

$$\frac{\text{O.D. (37°C)} - \text{O.D. (4°C)}}{\text{O.D. (37°C)}} \times 100 \quad (1)$$

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Aldehyde measurements

Another aliquot of the purified neutral salt-soluble collagen from each pooled sample was analyzed for the aldehyde content by the method of Paz *et al.*, (1965) and the results were expressed as μ moles of acetaldehyde equivalents/100 mg. of collagen.

Disc electrophoresis

Aliquots of the six samples of purified neutral salt soluble collagen were thoroughly mixed and lyophilized. The amount of α- and β-chain was estimated from this lyophilized sample by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels using 5% gels as described by Furthmayr and Timpl (1971). A suitable amount (0.25 mg) of collagen in 0.1 ml of sample buffer was heated at 50°C for 1 h and then 20 μl was applied to a gel. The electrophoresis was continued for 5 h at 6mA/tube. Fixation and staining were done using Coomassie brilliant blue and destaining by using 7% acetic acid. Then the gel column was loaded on a Joyce Loebl densitometer for scanning the bands. The amount of α- and β-chain were calculated from the ratio of areas on the graph.

Solubility of insoluble collagen in denaturing agents

The samples of skins after NaCl extraction were extracted thrice with 0.5 M citrate buffer (pH 3.6) to remove acid-soluble collagen and the residue was washed thoroughly with distilled water and stored at 4°C. This insoluble collagen (900 mg. wet wt.) from each sample was treated with 2 M KCNS or 6 M urea by the method of Adam *et al.* (1968).

Susceptibility of insoluble collagen to pronase

Another 500 mg (wet wt.) of insoluble collagen from each sample was treated with pronase according to Adam *et al.*, (1968). The amount of collagen solubilized in each case at varying intervals of time is shown in Fig. 1.

Statistical procedures

The results were statistically evaluated using Student's t test. The differences were regarded as significant at the level of $P < 0.05$.

RESULTS

The growth rate of the animals with protein malnutrition was slower than that of control animals. The loss of body weight was greater in younger protein-deficient animals compared to older animals.

A significant increase in the reversibility of neutral salt-soluble collagen was observed in protein deficient animals compared to controls (Table 1). The greater significance was found in younger protein deficient animals than older animals. The reversibility of neutral salt-soluble collagen decreased with aging (Table 1). The solubility of insoluble collagen in KCNS and 6 M urea increased significantly in protein deficient animals compared to controls (Table 2). The greater significance was found in younger deficient animals than older animals. The susceptibility of insoluble collagen in KCNS and 6 M urea decreased in age. An increased susceptibility of insoluble collagen to pronase digestion was observed during protein malnutrition but decreased in aging (Fig. 1).

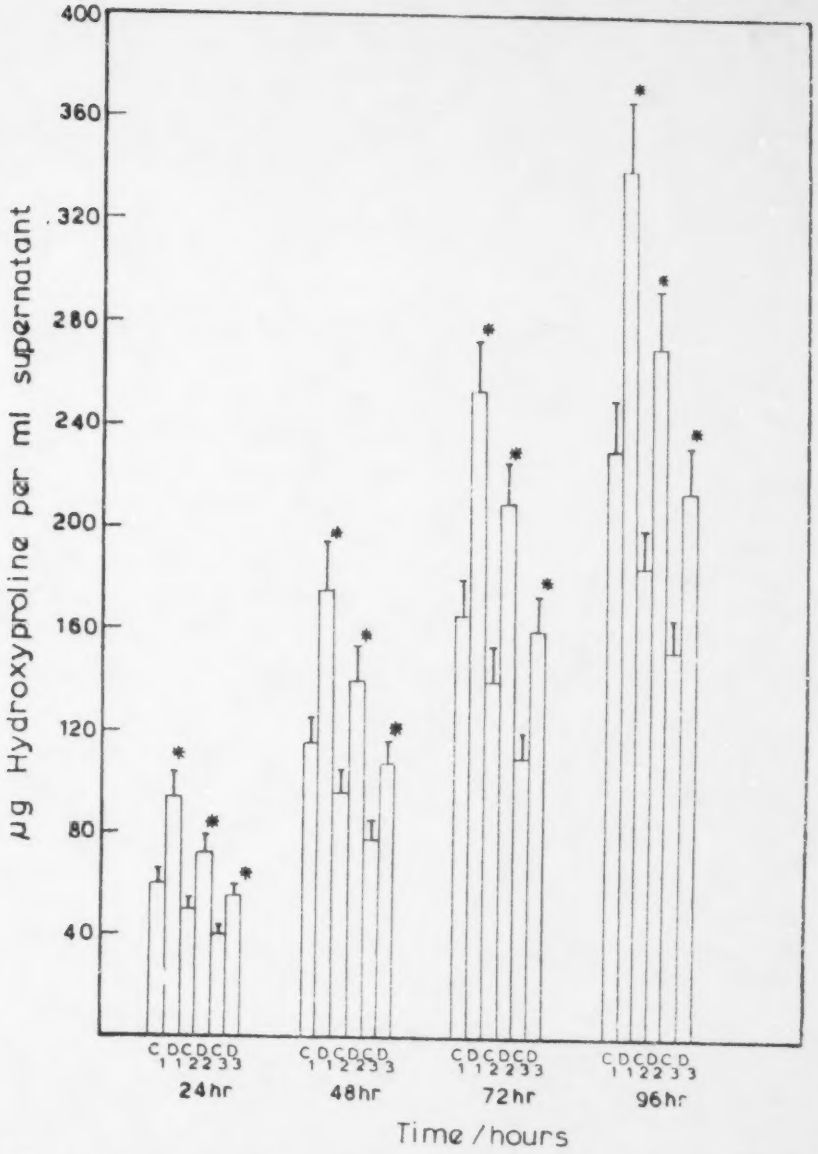

FIG. 1. Pronase treatment of insoluble collagen from: C_1 = 3 weeks control; C_2 = 10 months control; C_3 = 20 months control; D_1 = 3 weeks protein-deficient; D_2 = 10 months protein-deficient; and D_3 = 20 months protein-deficient groups. Values are mean ± S.D. and $P < 0.05$.

The aldehyde content of dermal neutral salt-soluble collagen was decreased significantly in protein deficient animals compared to controls (Table 3). The aldehyde content of dermal neutral salt-soluble collagen was decreased significantly in aging and the effect was greater in younger protein deficient animals than older ones.

The electrophoretic mobility of the α and β-chains of dermal neutral salt-soluble col-

TABLE 1. INFLUENCE OF PROTEIN MALNUTRITION ON THE PERCENT REVERSIBILITY OF DERMAL NEUTRAL SALT-SOLUBLE COLLAGEN GEL.*

Age	Group	Reversibility (percentage)
3 weeks	C	48.50 ± 3.9
	D	67.25 ± 5.2†
10 months	C	39.16 ± 3.0
	D	49.64 ± 2.5†
20 months	C	27.12 ± 2.1
	D	32.51 ± 1.92†

*Values are mean ± SE.
C = control; D = protein deficient.
† = $P < 0.05$ is considered to be statistically significant.

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lagen on SDS-polyacrylamide gels revealed four subunits, two monomers α_1 and α_2 and two dimers β_{11} and β_{12}. The densitometric patterns of control and protein deficient salt-soluble collagen are shown in Fig. 2. From Table 4 it is observed that the α-chains are increased significantly in protein deficient animals as compared to controls, whereas the β-chains are decreased significantly. The ratio of α/β chain was increased in protein-deficient animals but was decreased in aging. It is observed that α-chains are decreased and β-components are increased in aging.

DISCUSSION

The formation of gels from neutral salt-soluble collagen and its stability to cold have been found to be related to the degree of crosslinking in collagen (Gross, 1958). It has been reported by Adam *et al.* (1968) that the solubility of insoluble collagen to denaturing agents and pronase can be used as an indicator of collagen crosslinking. The results showed that dermal structural stability decreased in protein malnutrition but increased with aging.

The initial step in the crosslinking of collagen involves the oxidative deamination of the

TABLE 2. INFLUENCE OF PROTEIN MALNUTRITION ON THE SOLUBILITY OF INSOLUBLE COLLAGEN IN KCNS AND UREA.*

Age	Group	Solubilized collagen mg/ml	
		2 M KCNS	6 M urea
3 weeks	C	1.65 ± 0.12	1.87 ± 0.17
	D	2.41 ± 0.19†	3.12 ± 0.24†
10 months	C	1.31 ± 0.09	1.45 ± 0.11
	D	1.82 ± 0.15†	2.20 ± 0.19†
20 months	C	0.95 ± 0.08	1.05 ± 0.08
	D	1.22 ± 0.06†	1.45 ± 0.06†

*Values are mean ± SE.
C = control; D = protein deficient.
†$P < 0.05$ is considered to be statistically significant.

TABLE 3. INFLUENCE OF DIETARY PROTEIN LEVEL ON THE ALDEHYDE CONTENT OF NEUTRAL SALT-SOLUBLE COLLAGEN IN ALBINO RAT SKINS.*

Age	Group	Aldehyde content
3 weeks	C	0.66 ± 0.09
	D	0.56 ± 0.07†
10 months	C	0.51 ± 0.04
	D	0.44 ± 0.03†
20 months	C	0.38 ± 0.03
	D	0.34 ± 0.02†

*Values are mean ± SE. There are six samples with six skins in each sample.
C = control; D = protein deficient.
†$P < 0.05$ is considered to be statistically significant.

ϵ-NH_2 group of certain lysine or hydroxylysine residues by a copper dependent enzyme, lysyl oxidase (Bailey and Robins, 1973). The intramolecular crosslinks are formed by aldol condensation of two aldehydes (Tanzer, 1973). The intermolecular crosslinks are the products of a condensation reaction between one aldehyde and an ϵ-NH_2 group of lysine or hydroxylysine (Tanzer, 1973; Bailey *et al.*, 1974). It is interesting to note that infants with protein calorie malnutrition are found to have low liver and serum copper concentrations (Lahey, 1957; Mac Donald, 1961; Sandstead *et al.*, 1970). A decrease in the number of reducible crosslinks in collagen with age is probably due to the conversion of aldimine bond to a more stable non-reducible form (Robins and Bailey, 1972).

Previous studies of CM cellulose from this laboratory (Prasad and Bose, 1974) have indicated that α_1 and α_2 chain subunits of neutral salt-soluble collagen are appreciably increased and β_{11} and β_{12} chains are significantly decreased in protein deficient animals compared to controls. The yield of intramolecularly crosslinked β-components is also found to be reduced in protein deficient animals (McClain *et al.*, 1975). The neutral salt-soluble collagen, which is very rich in α-components decreases markedly with age (Orekhovitch *et al.*, 1960; Piez *et al.*, 1961; Butzow and Eichhorn, 1968).

The significant decrease in the percent of reversibility of dermal neutral salt-soluble collagen gels, decreased susceptibility of insoluble collagen in KCNS, urea and pronase, and the changes in the subunit pattern of neutral salt-soluble collagen collectively show that both intra- and inter-molecular crosslinkings of collagen were considerably increased in aging but impaired in protein malnutrition.

TABLE 4. EFFECT OF PROTEIN MALNUTRITION ON THE SUBUNIT COMPOSITION OF DERMAL NEUTRAL SALT-SOLUBLE COLLAGEN.

Age	Group	α_1	α_2	β	α/β
3 weeks	C	48	26	29	2.55
	D	53	30	16	5.19
10 months	C	40	21	34	1.79
	D	46	25	21	3.38
20 months	C	31	16	38	1.23
	D	36	21	27	2.11

C = control; D = protein deficient.

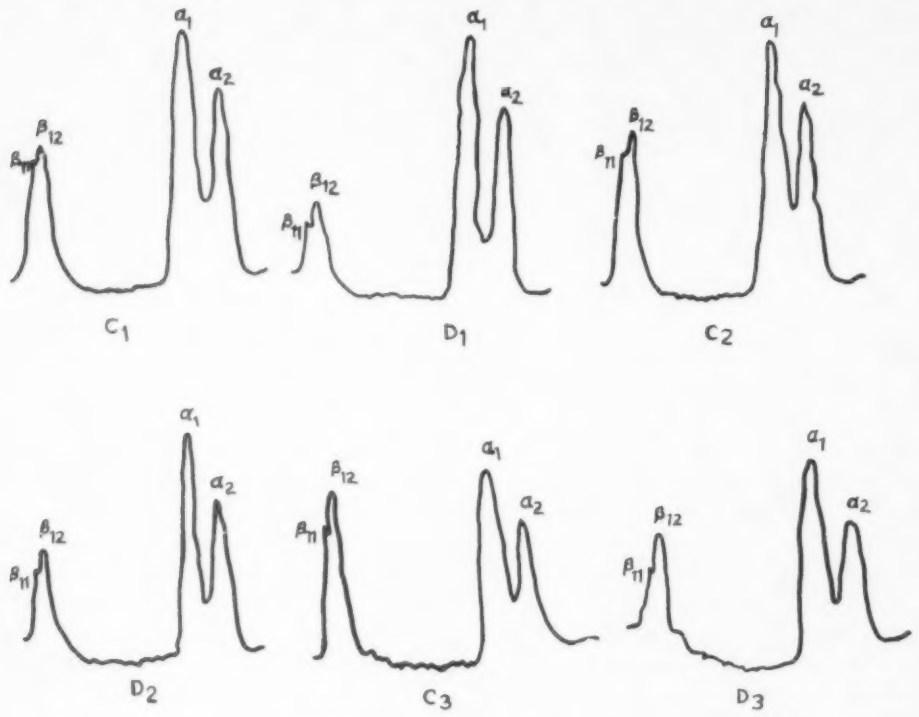

FIG. 2. Densitometer tracing of disc gel patterns obtained from dermal neutral salt-soluble collagen: C_1 = 3 weeks control; C_2 = 10 months control; C_3 = 20 months control; D_1 = 3 weeks protein-deficient; D_2 = 10 months protein deficient; and D_3 = 20 months protein deficient.

SUMMARY

The effect of protein malnutrition on collagen crosslinking was studied in different age groups of rats. The percent of reversibility of neutral salt-soluble collagen gel and the susceptibility of insoluble collagen to denaturing agents and pronase decreased with aging. The electrophoretic pattern of neutral salt-soluble collagen on sodium dodecyl sulphate polyacrylamide gels showed a decrease of α_1 and α_2 subunits and an increase of β-components, resulting in a decrease of α/β ratio in aging. There was a significant decrease in the aldehyde content of neutral salt-soluble collagen during age. Protein deficiency was found to impair the crosslinking and the effect was greater in younger animals than in older ones.

Acknowledgements—The authors wish to thank Prof. M. Santappa, Director of the Central Leather Research Institute, for his keen interest and his permission to publish this work. The financial assistance to one of the authors (JSR) by the Council of Scientific and Industrial Research, New Delhi, is gratefully acknowledged.

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Experimental Gerontology, Vol. 17, pp. 235–241, 1982
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AGING AND UTERINE GROWTH DURING IMPLANTATION IN C57BL/6J MICE

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(*Received* 20 *September* 1981)

Abstract—Uterine growth during implantation was compared in C57BL/6J mice aged 3–7 mo vs. 11–12 mo. All mice had given birth to at least one previous litter. Older mice had smaller uteri during early gestation (3 to 10 days post coitum) as measured by DNA content, although wet weight and protein were generally similar in both age groups. However, there were no age effects on uterine growth during implantation, as measured by the increments of DNA, as well as protein and wet weight per implantation site (decidual swelling) on days 6–10. These data are discussed in terms of the major increase in fetal death and resorptions in older mice subsequently observed by day 12–13. Larger age-related impairment of the artificially-induced decidual response is also considered. We conclude that impairments of the artificially induced decidual response do not predict the extent of decidual responses during pregnancy in aging mice.

INTRODUCTION

DESCRIBED HERE are manifestations of uterine functions in aging C57BL/6J mice during early pregnancy, which unexpectedly indicate an absence of marked impairments in uterine growth during implantation. Despite striking depletion of ovarian oocyte numbers during aging (Jones and Krohn, 1961; Talbert, 1977), C57BL/6J mice as well as many other laboratory rodents continue to produce normal numbers of ova during the initial phase of fertility decline before 12 mo (Harman and Talbert, 1970; Talbert, 1977). The number of implantation sites (decidual swellings) is only slightly reduced (−15%) during early pregnancy (days 6–10 post coitum) in 12 mo old C57BL/6J mice, although there is a 50% reduction of fertility at term (Holinka *et al.*, 1979a). Preliminary evaluation of the decidual responses on day 6–10 did not reveal age differences in the wet weight per implantation site, despite a smaller total uterine weight (Holinka *et al.*, 1979a). However a few days later, by day 13–14, fetal death and resorptions were two- to threefold higher in older mothers. The causes of this failure are undoubtedly complex and remain to be established.

Firstly, the endocrine milieu of pregnancy may play a role in fetal losses. Plasma progesterone (P) was lower in older mothers before implantation, particularly on day 4

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(Holinka *et al.*, 1979a). Deficits of P in this phase are critical, as shown by the increase of fetal resorptions after injections of antibodies to P on day 3 (Raziano *et al.*, 1972). Studies of the experimentally-induced decidual response also show critical requirements for P (Finn and Porter, 1975). After implantation no deficits of P were detected, even in mice whose litters underwent complete resorption. No deficits of plasma estrogen were detected in older mothers (Holinka *et al.*, 1979b).

Secondly, the uterus itself is likely to contribute to the early phase of reduced fertility. A uterine contribution is implied by the greater implantation failure of blastocysts transplanted from young donors to older hosts (Talbert and Krohn, 1966; Gosden, 1979) and by the widely observed impairments of the artifically induced decidual response of the uterus, which in young rodents closely resembles the normal decidual responses during implantation (Finn and Porter, 1975). For example, impaired decidual responses are observed in 9–12 mo old C57BL/6J mice, first ovariectomized and hormonally primed to simulate the milieu of pseudopregnancy, and then given an artificial decidualizing stimulus of either intrauterine oil (Shapiro and Talbert, 1974; Holinka and Finch, 1977) or intrauterine thread (Shapiro and Talbert, 1974). Similar impairments of artificially induced decidualization were observed in other mouse strains (Finn, 1966a; Gosden, 1979), rats (Chatterjee and Mukherjee, 1974), and hamsters (Blaha, 1967). In most reports, the increased uterine weight of the induced decidual responses in aging rodents is less than 25% of that in the young. Similiar impairments occurred in the increments of uterine glycogen, alkaline phosphatase, and DNA (Holinka and Finch, 1977). Such changes have lead to the widely held view that uterine aging is a major factor in rodent reproductive decline, as discussed for example by Pollard and Finn (1974) and Gosden (1979).

Despite the attention given to age changes in the induced decidual response, less is known about effects of age on the "natural" decidual response during implantation. Some reports suggest that the decidual swellings were of normal size (Blaha, 1970; Maibenco and Krehbiel, 1973). However, in hamsters aged 14–20 mo the initiation of decidualization was delayed by 12 hours and fewer decidual cells were formed during early implantation (Parkening and Soderwall, 1973; Thorpe and Conners, 1975). By 13–15 mo in hamsters, most decidua on day 8 are smaller and have diminished hyperemia in the stroma around the conceptus (Sorger and Soderwall, 1981), although some decidua are histologically normal. Differences in age-related changes between the artificially-induced decidual response and decidualization at implantation in earlier stages of reproductive aging were suggested by studies on 11–12 mo old C57BL/6J mice, in which the increase of wet weight per uterine swelling on gestation days 6–10 was not impaired (Holinka *et al.*, 1979a). This result apparently contrasts with the impaired artificially induced decidual responses of 9–12 mo old C57BL/6J mice (Shapiro and Talbert, 1974; Holinka and Finch, 1977). However, it is difficult to quantitatively compare the increase per swelling in decidua formed artificially with those of pregnancy, because artificial decidua often have irregular shapes and because they do not reflect the embryonic stimulus. Moreover, uterine wet weight may give a misleading impression about uterine growth because of possible age variations in tissue fluids, such as the pronounced stromal edema characteristic of early pregnancy (Finn and Porter, 1975), which appears to become variable during aging in at least one rodent, the hamster (Sorger and Soderwall, 1981). We therefore measured uterine DNA and protein during implantation in mice from this study (Holinka *et al.*, 1979a) to more directly estimate uterine growth.

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MATERIALS AND METHODS

C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice had at least 1 litter (3–7 mo old, uniparous; 11–12 mo old retired breeders) before being bred for this study with C3HeB/FeJ males. Day 1 of gestation was the first day after exposure to a male when a vaginal plug was observed. The same mice were studied previously (Holinka *et al.*, 1978; 1979a,b); their uteri were removed, blotted to remove surface and luminal fluids, weighed, and stored at −70 °C for 18 mo. From these, 40% were randomly sampled; uteri with ≤2 implantation sites were then excluded. The uterine weights and increase per decidual swelling of this sample (n = 125) were indistinguishable by ANOVA from values of the larger group (Holinka *et al.*, 1979a).

Uteri were homogenized in cold 0.25 M perchloric acid (PCA) and centrifuged for 10,000 g/10 min at 0 °C. The pellet was washed once with cold 0.25 M PCA; the DNA was then hydrolyzed and extracted with 0.5 M PCA (80 °C/15 hr) and estimated by the diphenylamine reaction (Burton, 1956) with calf thymus DNA as a standard. Protein was measured by the Folin phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin as a standard. Statistical significance was evaluated by analysis of variance (ANOVA) and the "student" Newman-Keuls procedure.

RESULTS AND DISCUSSION

Uterine wet weight differed between the age groups 3–7 mo vs. 11–12 mo only on day 7 (Fig. 1). Both age groups had similar time courses of weight change, with significant increases by day 8. Total uterine DNA was 10–15% smaller in the older mice, with significant differences on days 1, 3, and 7; changes were parallel in both age groups with significant increases by day 8. The net increment of DNA per uterus after day 3 was indistinguishable between the age groups. Total uterine protein on gestation days 1–8 was not altered by age, except on day 8.

Uterine growth after implantation (day 6–10) was also evaluated as the increase of uterine wet weight, DNA, and protein per implantation site (decidual swelling). The mean values of each age group on day 3 (just before implantation when these parameters are lowest) were subtracted from the individual values of day 6–10; the numeric remainder was divided by the number of implantation sites to estimate the increase per site. This calculation normalizes for the 10–15% fewer sites per uterus observed in 11–12 mo old mice on day 6–10 (Holinka *et al.*, 1979a) and for the smaller initial values in wet weight and DNA of older mice (Fig. 1). The increase of weight, DNA, and protein per implantation site on day 6–10 was similar in both age groups (Fig. 2).

The absence of age changes in the increase of DNA per site on gestation days 6–10 in C57BL/6J mice generally agrees with the previously reported similarity of changes in uterine wet weight and morphology of the larger sample of these same uteri (Holinka *et al.*, 1979a). Because both progesterone and estradiol are required for uterine decidualization and growth (Finn, 1966b; Psychoyos, 1967), the steroid milieu in the 11–12 mo old mice apparently sufficed, despite deficits of plasma progesterone before implantation (see Introduction). Decidual responses remain to be studied in older C57BL/6J mice during pregnancy; the possibility of impairments at later ages is suggested from observations on 13–15 mo old hamsters, whose decidua on day 8 are generally smaller (Sorger and Soderwall, 1981).

The implied absence of impaired capacity for decidual cell replication in 12 mo old mice is consistent with a previous histological study on steroid-stimulated endometrial stromal cell proliferation in 20 mo old mice of another strain (Finn and Martin, 1969). Thus, the repeated waves of uterine mitosis (Marcus, 1974) during the successive estrous cycles (about 10*) and pregnancies (about 5) experienced by these multiparous, 12 mo old mice did not exceed any limit of cell replication, such as exists in the hormone-dependent cell

*_Note added in proof:_ In the absence of pregnancy and under single housing conditions (optimal for short cycles) mice will have 40–50 estrous cycles during their reproductive lifespan.

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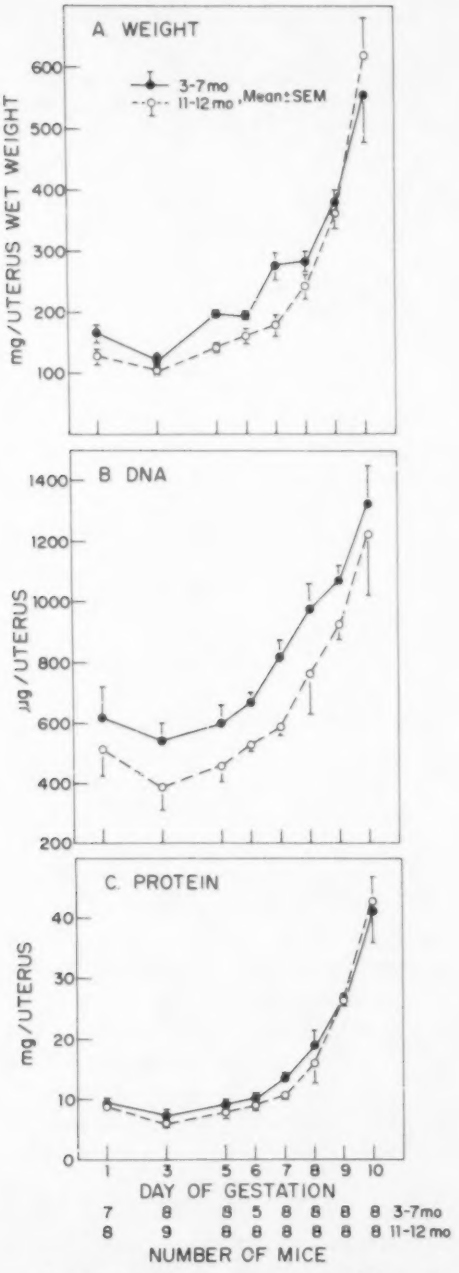

FIG. 1. Total uterine wet weight, DNA, and protein. The number of mice sampled on each day is given in the legend. By ANOVA, age differences were significant only in DNA ($p < .05$). By the Newman-Keuls test age differences were significant ($p < .05$) on the following days: weight, day 7; DNA, day 1, 3, 7; protein, day 8.

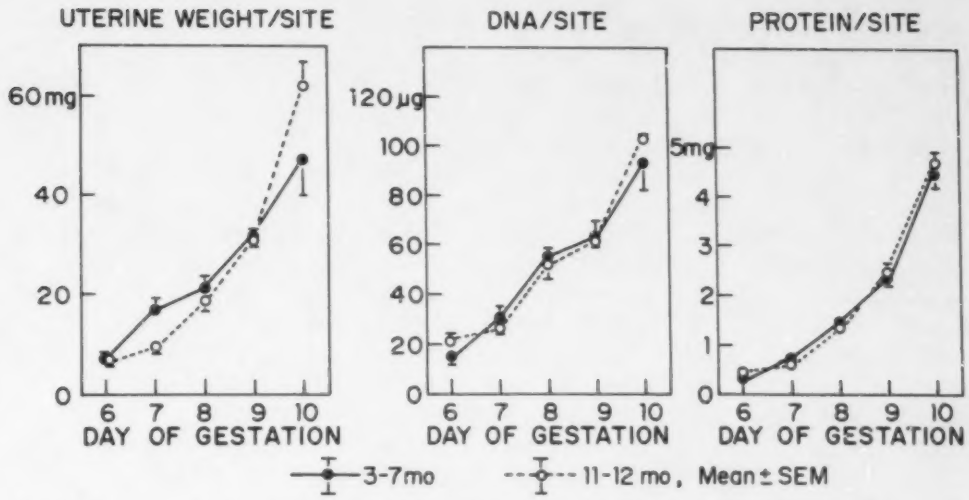

FIG. 2. Changes per implantation site (decidual swelling) in wet uterine weight, DNA and protein. The average weight on day 3 was subtracted from individual values on day 5–10; this remainder was then divided by the number of implantation sites to estimate the increase per decidual swelling. For number of mice, see Fig. 1. No significant age differences were detected by ANOVA or Newman-Keuls on any day.

cycles of the mammary alveolar epithelium (Daniel, 1977). Moreover, the failure of old mice to sustain the artificially induced decidual response was not altered by ovariectomy at puberty (Pollard and Finn, 1974).

The normal appearence of decidua during pregnancy in these 11–12 mo old mice contrasts with impairments of the artificially induced decidual response widely observed in mice aged 9–12 mo from diverse strains and reproductive histories: C57BL/6J (Shapiro and Talbert, 1974; Holinka and Finch, 1977), and CBA/Ca and CFLP (Gosden, 1979). (See Introduction for studies in other strains and species over a larger age range.) It seems striking that the artificially induced responses are so impaired by 9 mo in some mouse strains, in contrast to the gradual decline of fertility, which results initially from postimplantation losses. In the case of C57BL/6J mice, the oil-induced decidua were more than 50% smaller by 9–10 mo, whether the mice were multiparous (Shapiro and Talbert, 1974) or virgin (Holinka and Finch, 1977). In contrast, multiparous C57BL/6J mice had a postimplantation loss of fetuses which was 9% in young controls and 23% at 9 mo (Talbert, 1971). This dichotomy between the relatively abrupt effects of age on induced decidua vs. the gradual decline of fertility was also noted by Shapiro and Talbert (1974). The similar age change of induced deciduation in 9–10 month virgin or multiparous C57BL/6J mice in another study and the absence of effects from long term ovariectomy (Pollard and Finn, 1974) suggest that reproductive history has a limited influence on the extent of the oil-induced decidual response. Additionally, it is possible that the extent of the decidual response does not always bear on subsequent fetal viability, since on rare occasion a rodent fetus can develop normally without any decidual response, as in ectopic abdominal pregnancy with implantation in the omentum (Gosden and Russell, 1981).

Despite the apparently normal uterine growth during implantation in 11–12 mo mice of the present study, there was a major increase of fetal death and resorptions a few days later, after gestation day 12. Litter size at term was reduced to about 50% of the initial number of implantation sites. Additionally, litters varied considerably in the extent of

loss: In 6% of the older mice, the entire litter was resorbed (Holinka *et al.*, 1979a), an outcome which fell outside of a statistically normal distribution (Gosden *et al.*, 1981). There was no early indication of these embryonic deaths during implantation, as indicated by normal gross appearance of the decidua and by the similar statistical variances of wet weight, DNA, or protein per site in both age groups. However, gross measurements of decidual composition cannot identify age changes occuring in specific cell populations. Since decidua of normal size are induced in older hamsters by the more traumatic knife scratch procedure (Blaha, 1977), the threshold for inducing the full decidual response could change with age. Alternatively, in the early phases of reproductive senescence when most litters maintain normal growth of the non-resorbing fetuses (Holinka *et al.*, 1979a), abnormalities in ova prior to implantation may be important. For example, the age-related increase of abnormal meiosis (Henderson and Edwards, 1968; Crowley *et al.*, 1979) could contribute to the subsequent increase of fetal aneuploidy and other congenital defects (Fabrikant and Schneider, 1979). The random distribution of resorption sites in aging mice after day 12 (Gosden *et al.*, 1981) could result from random implantation of defective zygotes which can still induce normal decidua, but which fail developmentally and are subsequently resorbed. In such a case, defective ova might result from delayed ovulation with impairments arising in hypothalamic-pituitary functions (Finch *et al.*, 1980). Alternatively, there is evidence that persistent uterine scars from implantation can accumulate during successive pregnancies and may limit the number of implantations which the uterus can sustain (Gosden, 1979). In any case, the major impairments of the artificially induced response with age clearly do not predict the extent of the decidual growth response during implantation.

Acknowledgements—The measurements of DNA and protein were made by Heinz H. Osterburg, whose skills and thoughtfulness we gratefully acknowledge. We thank Kevin Flurkey for critical comments on this manuscript and Julie Patterson for assistance in statistical analysis. C.F.H. held a Postdoctoral Fellowship from the Population Council (N.Y.). This research was supported by grants to C.E.F. from the NIA (AG 00446, AG 00117, AG 007670) and NSF (PCM 76-02168).

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Experimental Gerontology, Vol. 17, pp. 243–244, 1982
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LETTER TO THE EDITOR

Sir,

I HAVE read the article entitled "Influence of aging on the surface area of the small intestine in the rat" (1981, *Experimental Gerontology* **16**, 399) with great interest. No doubt the authors, H. Meshkinpour, M. Smith and D. Hollander, are excellentiy competent to choose one of the more intriguing problems in the physiology of the gastrointestinal tract: how to correctly quantify the intestinal response, *cf.* absorption, in aging. It has truly been a challenge for many years to express the data which describe the alterations in absorption from the aging gut.

However, as a concerned person let me add some remarks on this report. I am not at all convinced that the method applied is the most appropriate. More exactly, I do not think that the present procedure for measuring gut length is the best way (at least in such a manner) to estimate the actual size, moreover the surface area, of the small intestine. The thing is that one cannot draw conclusions from the suspended intestine with a constant weight attached to it if the samples are from animals of different ages. The reason is that the ratio of the active functional part of the intestine compared to the non-active components (connective tissue, blood- and lymph vessels, etc.) is not constant during aging. It is well known that there are great differences in the increase of connective tissue and amyloid-like substances even within a species, i.e. C57B1 and SWR/J mice strains (Andrew and Andrew, 1957; Moog, 1977). This leads to the fact that the extension of gut with a constant weight will considerably differ in the various age groups of animals, not

TABLE 1. COMPARISON OF LENGTHS OF THE SMALL INTESTINE MEASURED HORIZONTALLY (UNLOADED) AND VERTICALLY (LOADED WITH CONSTANT WEIGHT).

Age (weeks)	Body weight (g)	Intestinal length (cm) "A"	Intestinal length (cm) "B"
10.8	190 ± 18 (30)	64,7 ± 7,3 (30)	111,8 ± 6,4 (30)
26	221 ± 14 (34)	62,1 ± 8,1 (34)	114,6 ± 5,1 (34)
52	235 ± 25 (30)	63,6 ± 8,2 (30)	112,0 ± 5,9 (30)
116	260 ± 40 (30)	63,6 ± 9,7 (30)	128,4 ± 10,1 (30)

Mean ± s.d. (*n*)
"A" = intestinal length measured horizontally (in unloaded condition).
"B" = intestinal length measured vertically (loaded with constant weight).
The animals were Wistar female rats of OÉTI strain fed constant laboratory chow (LATI, Gödölló, Hungary).

because the length of the small bowel would largely increase with aging, but because the proportion between the structural elements alters during the lifespan.

We have also done such measurements on the rat's small intestine together with the measurement of (unfixed) gut positioned horizontally on a wetted plastic sheet. The differences are shown in Table 1. The absolute length of the small intestine does not increase within the range investigated. This is true for males as well, according to which the absolute (unfixed) length of the small gut increases up to 150 g body weight and then remains fairly constant (Pénzes and Skála, 1977).

It must be mentioned that not only the present authors have estimated the size of the small gut in such a way but Klimas (1968) has done so as well. He has observed a similar increase in gut length with aging in the rat. The present objection is valid even if one has to consider that Meshkinpour *et al.* have done their observations on perfused and prefixed intestine.

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Experimental Gerontology, Vol. 17, pp. 245–254, 1982
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BIOCHEMICAL EVENTS ASSOCIATED WITH LYMPHOCYTE ACTIVATION IN AGING

K^+ Transport and Sensitivity of the Na^+-K^+ Pump to Digoxine

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(*Received* 20 *September* 1981)

INTRODUCTION

THE MECHANISM by which *in vitro* and *in vivo* T cell responses decline with age (Makinodan, Good and Kay, 1977; Kishimoto, Tomino, Inomata, Kotegawa, Saito, Kuroki, Mitsuya and Hisamitsu, 1978) has not already been established. Works based on the identification of blood T-lymphocyte subpopulations determined by various surface markers report only minor, if any, age-related changes (reviewed by Leech, 1980). According to different experimental conditions, both decline or enhancement in helper or suppressor T cell modulator functions could be observed (Hallgren and Yunis, 1977; Antel, Weinrich and Arnason, 1978b; Kishimoto, Tomino, Mitsuya and Fujiwara, 1979).

These findings argument the hypothesis of a qualitative defect common to T lymphocytes in aging. Indeed, recent reports have shown that the decreased DNA synthesis by phytohaemagglutinin (PHA) stimulated peripheral blood lymphocytes (PBL) in old humans results from both a reduced number of activated T lymphocytes and from an impairment in the proliferative potential of initially responsive cells (Hefton, Darlington, Casazza and Weksler 1980).

In spite of the numerous works on immunosenescence topics, little is known concerning the early biochemical events associated with T lymphocyte activation in the elderly while studies of these processes might provide interesting information.

This work was supported by a grant from the "Fonds Interuniversitaire de Recherche pour le Processus du Vieillissement."

Part of this work has been presented at the poster session of the XXIXth Colloquium, Protides of the Biological Fluids, May 4–7, 1981, in Brussels.

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There exists a normal (Hung, Perkins and Yang, 1975) or a moderate increase (Callar and Basten, 1977) in the number of affinity of the PBL membrane receptors to PHA and the cell kinetic seems to be unaltered during lymphocyte activation in the elderly (Hefton *et al.*, 1980; Callar and Basten, 1977). Therefore, a decreased intrinsic capacity to transmit activation signals or to respond to it should be considered.

A previous work emphasized the integrity of early Ca^{2+} uptake, a consequence of PHA-induced increase of membrane permeability and considered as a "general cell-signalling mechanism" of cell activation (Kennes, Hubert and Nève, 1981). The aim of the present study is to specify another early membrane dependent event, the early increase of K^+ fluxes induced by mitogenic concentration on PBL from the elderly. Indeed, changes in membrane permeability to K^+ and activation of the membrane associated Na^+, K^+-ATPase have been established as an early and essential step in lymphocyte activation (Hume and Weidemann, 1980). The sensitivity to digoxine, an ouabain-like Na^+-K^+ pump antagonist, is also tested in PHA-stimulated PBL from the elderly.

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METHODS AND PROCEDURE

Subjects

Blood was collected from 20 old (over 70 years of age) and 20 adult (between 20 and 35 years of age) subjects. All of them were healthy and untreated.

Preparation of lymphocytes

Peripheral venous blood was drawn in syringes containing calparine. After one hour of incubation at 37 °C, the mononuclear cells were isolated by centrifugation over Histopaque 1077 (Sigma) and washed three times in Ca^{2+} and Mg^{2+} free Hanks' (Flow Laboratories). They were immediately used for cell cultures. The mononuclear cell suspensions from the two groups of subjects contained approximatively 70% E-rosette forming cells and 20% Ig-bearing cells.

Measurement of thymidine incorporation

The procedure has been previously reported (Kennes, Hubert, Brohée and Nève, 1981).

The PBL were cultured without (resting) or with optimal doses of PHA (final concentration 10 μg/ml) added at the start of the culture.

Digoxine (Welcome) was dissolved in RPMI 1640 and 100 μl of the solution was added to 900 μl of culture medium to obtain the desired final concentration. These concentrations did not inhibit the spontaneous DNA synthesis of unstimulated PBL and toxic effects were excluded by the trypan blue exclusion test.

Rubidium influx

Fresh PBL: Immediately after their recount, the PBL were adjusted to 2.10^6/ml in culture medium (RPMI 1640 with glutamine 1 mM/ml, gentamycine 3 μg/ml and human serum 10%). Aliquots of 400 μl were then distributed in Eppendorf microtubes and 10 μl of RPMI 1640 with or without 4 μg PHA were added to appropriate tubes. Cells were incubated at 37 °C in a 5% CO_2 incubator. Thirty minutes before harvesting, the cultures were pulsed with 4 μCi (in 20 μl) of ^{86}RbCl (New England Nuclear). Cells were sampled using an adapted method from Malaisse *et al.* (1978). Two first centrifugations (20 sec., Beckman Microfuge Model 152) without resuspension were performed after supplements of respectively 600 μl (first) and 1000 μl (second) of ice cold Ca^{2+} and Mg^{2+} free Hanks' to deposit the cell in the tip of the tube and dilute the radioactivity of the medium. The supernatants were removed and Di-n-butylphtalate (1 ml; BDH Chemicals, Poole, England) was then layered on the top of residual medium and a third centrifugation (20 sec) performed to separate the PBL from aqueous phase. A last aspiration was done and the radioactivity of the pellet after resuspension in 200 μl of formic acid counted for 1 min. in the presence of 5 ml of Scintisol (Isolab) with a liquid scintillator counter.

Preincubated PBL: PBL were prepared as above, but the cultures (2 ml per tube) were maintained in capped tubes (Falcon) at 37 °C for 20 hours with or without PHA (10μg/ml). The cultures were then pulsed with 20 μCi (40 μl) of ^{86}RbCl and sampled at the indicated times described above.

The nature of $^{86}Rb^+$ (rubidium) influx was evaluated using DGX (25 μM) and furosemide (Fu: 3mM) (Hoechst) known to inhibit respectively the Na^+, K^+-ATPase pump and a Na^+-K^+ co-transport system (Garay, Dagher, Pernollet, Devynck and Meyer, 1980). Both drugs were dissolved and diluted into RPMI 1640 before use.

Conversion of the results in K^+ uptakes: It was performed according to Owens with equations (1) and (2):

$$SRM\ (\text{count/fmol } K^+) = \frac{\text{counts} \cdot \text{ml}^{-1}}{Z\ (\text{fmol} \cdot \text{ml}^{-1})} \quad (1)$$

where SRM = specific radioactivity of the culture medium and Z = the concentration of K^+ ($5{,}9 \times 10^9$ fmol • ml^{-1})

$$K^+ \text{ uptake } (\text{fmol} \cdot \text{cell}^{-1}) = \frac{\text{count in pellet} - \text{count in trapped medium}}{SRM \times \text{cells} \cdot \text{pellet}^{-1}} \quad (2)$$

Correction for the trapped space was done using the zero time uptake measured as follows: cells and K^+ tracer were precooled at 4 °C for 30 min., mixed rapidly and sampled following the method described earlier (Owens and Kaplan, 1980).

Rubidium efflux

Efflux was measured using preloaded PBL (20 hours in culture medium supplemented with $^{86}RbCl$ 10 μCi/ml, $2 \cdot 10^6$ cells/ml) and then washed three times in Ca^{2+} and Mg^{2+} free Hanks' before resuspension in a same volume of culture medium. Aliquots of 2 ml into capped tubes (Falcon) were incubated with or without PHA (10 μg/ml) for indicated times and the cultures were sampled as described above. Trapped medium exhibited a low radioactivity unable to significantly influences the measures.

Efflux was estimated by the equation $(1 - Pt/Po) \times 10^2$ where Pt and Po are cell tracer content (cpm/sample) at times t and zero respectively.

Curve fitting

Adjustment of experimental exponential curves to mathematical linear equation ($y = a + bx$) was done with program H.P. no. SD-03A on a Hewlett-Packard 97 calculator. Three or more triplicate points were used and coefficient of correlation was higher than 0.9 for each case.

Statistical analysis

Each experiment paired for age was realized in triplicate and the mean result (coefficient of variation less than 5%) used for statistical evaluations.

Comparisons between groups were done using the non parametric two-tailed Wilcoxon test for paired data.

RESULTS

Dose-related inhibitory action of digoxine on ^{3}H-Tdr incorporation by PHA-stimulated PBL

Increasing concentrations of digoxine (0,25 to 25 μM) progressively lowered the tritiated thymidine (^{3}H-Tdr) incorporation induced by optimal doses of PHA. No differences either in the inhibiting concentrations or in the slope of the dose related inhibitory curves ($p > 0{,}1$) could be found between old subjects (O) and adult subjects (A) (Fig. 1) despite the reduced ^{3}H-Tdr response of PHA-PBL O.

$^{86}Rb^+$ influx by resting and PHA-stimulated PBL

PHA (10 μg/ml) significantly increased the rate of $^{86}Rb^+$ uptake by PHA-PBL in both A and O ($p \leq 0.01$) (Fig. 2). A change of the monovalent cation transport was still detectable after 5 minutes of incubation (data not shown). The initial rate (a) of $^{86}Rb^+$ transport

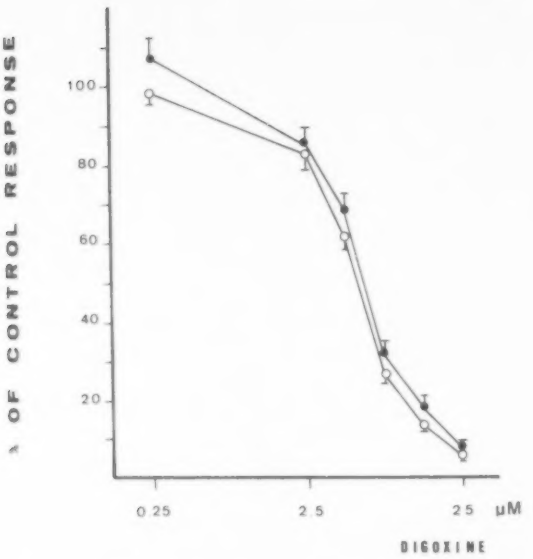

FIG. 1. Effect of digoxine on ^{3}H-Tdr incorporation by PHA-stimulated PBL. The lymphocytes from old • and adult o subjects were cultured with various concentrations of DGX added prior to PHA (10μg/ml) addition at the start of the 72 hour culture. Results are expressed in percentage of ^{3}H-Tdr incorporation by PHA-PBL control. (old: 94 ± 15 × 10^3 cpm. cult^{-1} ± sem; adult: 166 ± 20 × 10^3 cpm. cult^{-1} ± sem; $p \leq 0.01$, n: 11 paired experiments). After semilog plot of the curves to linear equation $ln\, y = a + bx$ where y = % of the control response and x = the DGX doses (μM), the parameters show in adult: $a = 4.68 \pm 0{,}18$; $b = -296 \pm 18$ and in old: $a = 4.75 \pm 0.23$; $b = -279 \pm 21$. No statistical differences could be found between a_A and a_O or b_A and b_O ($p > 0{,}1$).

in the elderly was slightly higher for fresh lymphocytes ($p \leq 0.1$) or significantly increased for preincubated lymphocytes ($p \leq 0.01$) when compared to the adult group. After PHA treatment, the rate of cation uptake by PBL O was statistically indistinguishable from PBL A.

Due to the difference between the basal activity of K^+ transport by resting-PBL O compared to that by resting-PBL A, an index of stimulation was calculated for each experiment as shown in Table 1. Results were absolutely comparable in both groups ($p \geq 0.1$).

The index changed from about 1.6 to 2 when PBL A were stimulated for 20 hours instead of 1 hour ($p \leq 0.01$).

$^{86}Rb^+$ efflux by resting- and PHA-stimulated PBL

As found for influx, PHA increased equally the $^{86}Rb^+$ efflux of PBL from both groups of subjects (Fig. 3). The analysis of the stimulation index showed superposable values in A

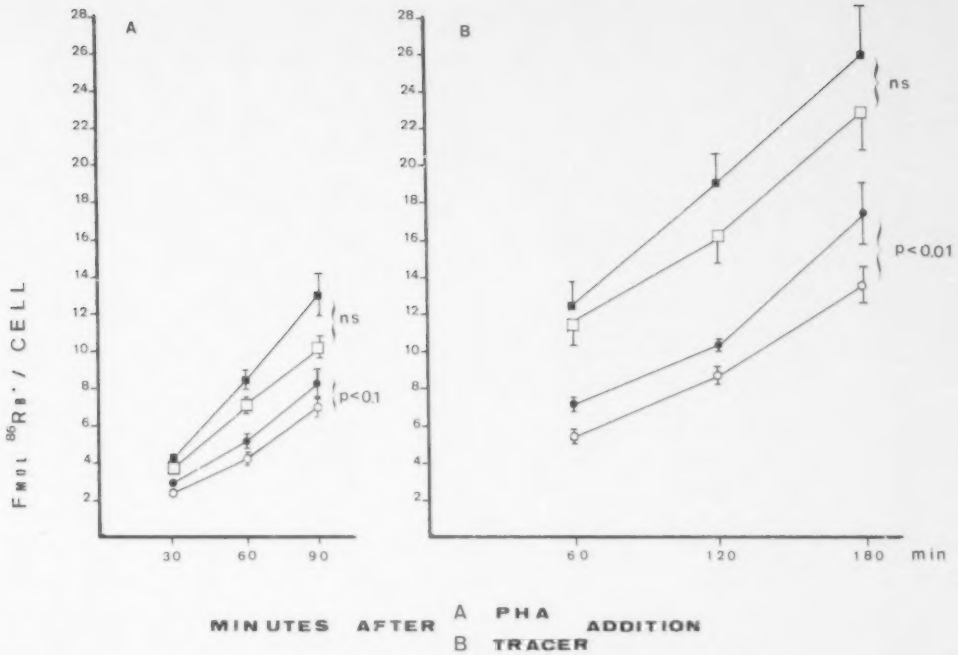

FIG. 2. $^{86}Rb^+$ Influx by resting- and PHA-PBL from adult (A) and old (O) subjects. O resting-PBL adult; □ PHA-PBL adult; • resting-PBL old; ■ PHA-PBL old. Ten paired experiments (± sem) were compared, each was performed in triplicate and the mean value used (standard deviation less than 5%). The uptake was tested as described in Materials and Methods.

A. $^{86}Rb^+$ Influx by fresh PBL. After semilog plots of the curves to linear equation $ln\ y = a + bx$ where y = $^{86}Rb^+$ uptake and x = time of incubation (min), it is found that PHA raises (a) in adult from 0.32 ± 0.05 to 0.75 ± 0.12 ($p \leq 0.01$) and in old from 0.54 ± 0.10 to 0.80 ± 0.08 ($p \leq 0.01$). PHA does not act on the slope of the curve (b) which was identical in A and O. Statistical comparisons between O and A show in resting PBL $a_O > a_A$ ($p \leq 0.1$) and in PHA-PBL $a_O = a_A$ ($p > 0.1$). $b_A = b_O$ for resting- and PHA-PBL ($p > 0.1$)

B. $^{86}Rb^+$ Influx by 20-hour preincubated PBL with or without PHA. Semilog plots of the curves as described above indicate that PHA raises (a) in adult from 1.18 ± 0.06 to 1.97 ± 0.11 ($p \leq 0.01$) and in old from 1.39 ± 0.08 to 1.86 ± 0.11 ($p \leq 0.01$). In resting PBL $a_O > a_A$ ($p \leq 0.01$) and in PHA-PBL $a_O = a_A$ ($p > 0.1$). The slope (b) remains unchanged and identical in A and O.

and O which were in the same range from those observed for K^+ influx by early activated PBL (Table 2).

Inhibitory effect of digoxine and furosemide on $^{86}Rb^+$ transport

The sensitivity of $^{86}Rb^+$ influx to digoxine (DGX), an inhibitor of the NA^+, K^+-ATPase enzyme, was tested with the aim of discovering which part of the enzyme is involved in the process studied. At a concentration that completely inhibited the ^{3}H-Tdr incorporation after 72 hours (Fig. 1), DGX (25 μM) added at the start of the incubation lowered the $^{86}Rb^+$ uptake by resting-PBL to about 50% of the normal value (Fig. 4). In addition, the glycosid abolished the triggering action of PHA on cation transport.

Furosemide (Fu:3 mM) added at the start of the culture induced a fall of about 30% but

TABLE 1. $^{86}Rb^+$ INFLUX BY PHA-STIMULATED PBL. INDEX OF STIMULATION IS CALCULATED USING THE EQUATION $(Rb\,u\,\int_{PHA})\,(Rb\,u\,\int_{r}^{t})^{-1}$ WHERE $Rb\,u\,\int_{PHA}$ = $^{86}Rb^+$ UPTAKE BY PHA-PBL AT TIME t AND $Rb\,u\,\int_{r}^{t}$ = $^{86}Rb^+$ UPTAKE BY RESTING-PBL AT TIME t. FOR DETAILS OF EXPERIMENTS SEE LEGEND UNDER FIG. 2.

	Index of stimulation (± sem)		
Time of incubation with PHA	Old	Adult	W
30 min.	1.37 ± 0.06	1.42 ± 0.04	ns
60 min.	1.55 ± 0.07	1.58 ± 0.06	ns
90 min.	1.57 ± 0.06	1.53 ± 0.07	ns
24 hrs.	1.69 ± 0.12	2.03 ± 0.12	ns

W = non parametric two-tailed Wilcoxon test for paired data; $n = 10$; ns = $p > 0.1$.

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did not affect the flux ratio between activated and non activated fresh PBL. The inhibitory effects of both drugs were cumulative since more than 80% of the transport was suppressed in their simultaneous presence.

DISCUSSION

$^{86}Rb^+$, a congener of K^+, has been demonstrated as a useful tracer of K^+ movements (Bonting, 1970; Hart, 1981) and the analysis of $^{86}Rb^+$ transport on lymphocytes shows that with optimal concentrations for PBL-DNA synthesis, PHA induces a 1.52- and 2-fold increase of K^+ uptake respectively 1/2 to 1 hour and 20 hours after the addition of the mitogen. The index value for early stimulation PBL is lower than that reported by Segel *et al.* (1,85 for the first hour after PHA addition) (Segel and Lichtman, 1976). However, this discrepancy should not change the comparison between the two populations studied in the data.

The increase of the stimulation index observed if cells are incubated for 20 hours more is interesting. The phenomenon can be correlated with a similar enhancement of cell water volume measured by ^3H_2O (data not shown) and thus probably depends on cell volume changes during blastogenesis (Labadie, Beyer, Harrison, Mitchell and Bowers, 1979).

DGX-sensitive Na^+-K^+ pump yields about 50% of the K^+ inflow, while 30% results from a Fu-sensitive co-transport, both effects being additive. These observations fit in with recent publications (Owens and Kaplan, 1979). Furthermore, identical doses of DGX counteract the PHA-induced increase of the K^+ influx and inhibit the DNA synthesis by mitogen-treated PBL, corroborating the essential role played by the Na^+, K^+-ATPase enzyme in the triggering mechanism of lymphocyte activation.

TABLE 2. $^{86}Rb^+$ EFFLUX BY PHA-STIMULATED PBL. INDEX OF STIMULATION IS DETERMINED BY THE EQUATION $(1 - Pt/Po)_{PHA} \times (1 - Pt/Po)^{-1}_{RESTING}$ WHERE Pt = $^{86}Rb^+$ CONTENT IN PELLET AT TIME X AND Po = $^{86}Rb^+$ CONTENT IN PELLET AT TIME 0 FOR RESTING OR PHA-PBL. FOR DETAILS OF EXPERIMENTS SEE LEGEND UNDER FIG. 3.

	Index of stimulation (± sem)		
Time of incubation with PHA	Old	Adult	W
60 min	1.42 ± 0.04	1.44 ± 0.04	ns
120 min.	1.36 ± 0.04	1.39 ± 0.08	ns

W = non parametric two-tailed Wilcoxon test for paired data; $n = 11$; ns = $p > 0.1$.

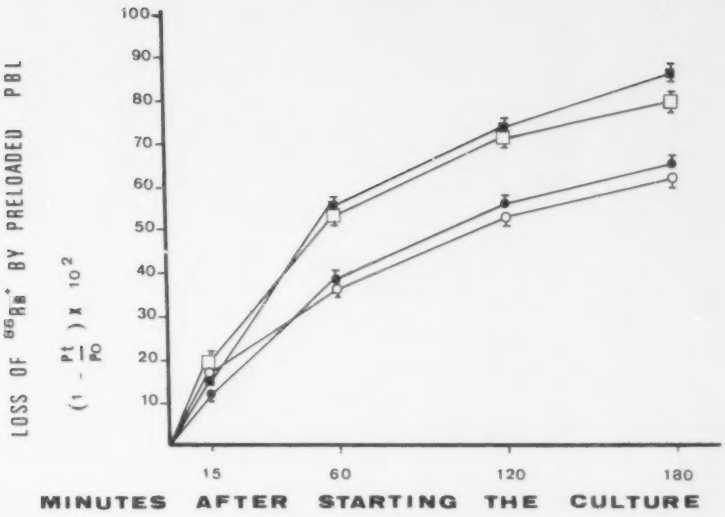

FIG. 3. $^{86}Rb^{+}$ Efflux by PBL from adult and old subjects. O resting-PBL adult; □ PHA-PBL adult; • resting-PBL old; ■ PHA-PBL old. Eleven paired experiments (± sem) were compared, each was performed in triplicate and the mean value used (standard deviation less than 5%). The experiments were done as described in Materials and Methods. The $^{86}Rb^{+}$ efflux was evaluated using the ratio: $(1 - Pt/Po) \times 10^2$. Pt = $^{86}Rb^{+}$ content in pellet at time x and Po = $^{86}Rb^{+}$ content in pellet at time o for resting- and PHA-PBL.

Semilog plots of the curves to $ln\ y = a + bx$ where $y = Pt.\ Po^{-1}$ and $x = t$ indicate that PHA raises (a) in adult from 3.33 ± 0.18 to 3.75 ± 0.03 ($p \leq 0.01$) and in old from 3.22 ± 0.07 to 3.71 ± 0.04 ($p \leq 0.01$) while (b) remains unchanged $a_O = a_A$ ($p > 0,1$) for resting- and PHA-PBL.

In agreement with previous findings of Segel and Lichtman (1976) who demonstrate an unchanged K^+ concentration during lymphocyte activation, PHA raises the K^+ efflux within the same range than for K^+ influx.

The role of K^+ movements on lymphocyte activation has only been stressed in recent years and is reviewed elsewhere (Hume and Weidemann, 1980). Briefly, the activation of plasma membrane Na^+, K^+-ATPase leading to increased K^+ influx, appears to be essential for commitment into the proliferation state (Hume and Weidemann, 1980) and is absolutely required for maintaining the Na^+/K^+ gradient through the plasma membrane in spite of the cell volume increase during blastogenesis (Lauf, 1975). Moreover, recent findings support evidence for the involvement of the active pump in the early triggering mechanism of lymphocyte activation (Segel, Androphy and Lichtman, 1978).

It has already been demonstrated that lymphocyte stimulation results from complex mechanisms including at least mitogen-induced alterations at the cell surface of early responding cells (Wedner and Parker, 1976) and the latter processes requiring cell-cell cooperation (Katz, Kierszenbaum and Waksman, 1978). Thus, questioning whether the age-related immune deficiency could result from one or more defects of these events, it seems useful to discriminate the different steps of the triggering processes leading to T-cell activation. Since plasma membrane Na^+, K^+-ATPase activation results from direct interaction between mitogen-receptor loaded and the enzyme (Hume and Weidemann, 1980) and owing to its essential role in cell proliferation, we focused on PHA-induced K^+

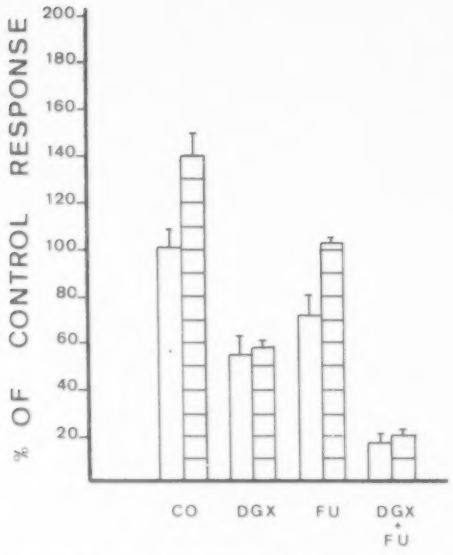

FIG. 4. Action of digoxine (DGX : 25 μM) and furosemide (FU : 3mM) on $^{86}Rb^+$ fluxes by resting- and PHA stimulated-PBL.

The drugs were added 15 min before PHA and $^{86}Rb^+$ Cl. The incubation was harvested 30 min after starting the culture. Results are expressed as the percentage of rubidium uptake by resting PBL obtained in the absence of the drugs. The means of four experiments performed in triplicate are shown.

fluxes in PBL from old subjects as a model for membrane reactivity to stimulation in aging.

The present study shows that PHA equally triggers early K^+ influx by PBL from O and A individuals. Because this lectin-induced K^+ influx increase is a consequence of Na^+, K^+-ATPase activity, the integrity of the pump activation mechanism has to be assumed in aging. Moreover, in spite of the depressed DNA synthetic response to PHA, a normal sensitivity to the DGX-inhibiting action on ^{3}H-Tdr incorporation by PHA-PBL is found in O. The latter observation suggests that digoxine sensitive structures work freely during PHA-induced lymphocyte proliferation in aging, thereby supporting further arguments for adequate Na^+, K^+-ATPase activity.

The K^+ outflow by freshly collected resting-PBL shows identical patterns in both categories of individuals. The efflux increases similarly with PHA, in agreement with a previous report where Ca^{2+} uptake was found to occur normally in aging (Kennes *et al.*, 1981). Indeed, both passive Ca^{2+} influx and K^+ efflux changes result from the enhancement of PBL plasma membrane permeability to these cations (Whitney and Sutherland, 1973; Freedman, 1979).

The foregoing bears evidence that early responding T-cells have a normal capacity to generate early membrane dependent events associated with T-cell activation in the elderly.

In contrast, although the statistical significance appears to be restricted to 20-hour pre-incubated cells, the K^+ influx shows an age-related increase in resting PBL O. Such

enhancement could result from a qualitative cell abnormality leading to an increase of Na^+, K^+-ATPase activity in PBL as observed in other mammalian tissues from old subjects (Hayflick, 1980) or might also reflect differences in the distribution of blood T-cell subpopulations committed in activated stage. Its significance for age-related immune deficiency remains unclear owing to the integrity of the active K^+ pump triggering mechanism in aging.

SUMMARY

The present investigation was directed toward answering the question of whether some age-related changes of membrane dependent triggering mechanisms during lymphocyte activation could account for the depressed T cell response to mitogens in aging.

For this purpose, the K^+ movements were analyzed in PHA-stimulated peripheral blood lymphocytes (PHA-PBL) from old humans (O) compared to adult (A). Indeed, plasma membrane Na^+, K^+, ATPase activation plays an essential role in cell proliferation and results from direct interaction between the loaded mitogen receptor and the enzyme.

No difference could be found in the magnitude and the timing of the PHA-induced increase of K^+ fluxes between PHA-PBL from O and A despite a higher K^+ inflow in unstimulated but 20-hour preincubated PBL from O. Further experiments showed that the lectin-induced triggering mechanism of cation transport resulted from digoxine (DGX: a glycosid cardiotonic) sensitive ATPase. Moreover, whereas PBL from O exhibited a decreased PHA-induced DNA synthesis, DGX depressed the thymidine incorporation by 72-hour cultured PHA-PBL within the same inhibitory dose-related pattern in both O and A.

We conclude that the triggering mechanism of Na^+, K^+-ATPase induced by PHA occurs adequately in early stimulated PBL from old subjects. In addition, digoxine sensitive structures work freely during PHA-induced lymphocyte proliferation in aging, thereby supporting further agruments for adequate Na^+, K^+-ATPase activity.

Acknowledgements—The authors gratefully acknowledge the expert technical assistance of Miss Pietrina Piro and Mr. Michel Devos, and thank Miss Hanotiau and Mrs. Kennes for typing the manuscript.

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Experimental Gerontology, Vol. 17, pp. 255–262, 1982
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THE EFFECT OF TEMPERATURE AND OF DIETARY RESTRICTION ON LIFESPAN AND REPRODUCTION IN THE ROTIFER *ASPLANCHNA BRIGHTWELLI*

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(*Received* 20 *September* 1981)

Abstract—In this study, the effects of different environmental temperatures and of dietary restriction on the lifespan, reproductive cycle, and fecundity of the rotifer *Asplanchna brightwelli* were examined. When temperature effects were analyzed, it was observed that the mean lifespan and the times at which the reproductive cycle started and ended all increased as environmental temperatures decreased. When dietary restriction was imposed by increasing the interval between feeding times from 12 to 36 hours, there was an increase both in the mean lifespan and in the length of the reproductive period in this rotifer.

INTRODUCTION

IN THIS study, temperature and diet were manipulated to determine their effect on longevity and reproduction in the rotifer *Asplanchna brightwelli*. The effects of different temperatures on lifespan have been studied using the rotifer *Philodina acuticornis odiosa*, Milne (Meadow, 1967; Fanestil and Barrows, 1965) as well as in other invertebrates, including *Drosophila* (Loeb and Northrop, 1917) and *Daphnia* (MacArthur and Baillie, 1929). These studies on poikilotherms showed that lifespan would increase as environmental temperature decreased, within physiological limits.

Food availability as a form of dietary restriction has also been studied extensively by researchers in the field of aging. In a wide range of laboratory animals, dietary restriction has been shown to increase longevity. This was demonstrated in invertebrates such as *Tokophyra* (MacKeen and Mitchell, 1975), *Campanularia flexuosa* (Barrows and Kokkonen, 1978), *Daphnia* (Ingle, Wood and Banta, 1937) and in *Philodina* (Fanestil and Barrows, 1967).

This study will show how different temperatures and various levels of dietary restriction affect lifespan and reproductive patterns in *A. brightwelli*. *A. brightwelli* is of interest for aging studies because of its very short 5 day mean lifespan. Its lifespan is shorter than that of any species cited above where the influence of temperature or diet on lifespan has been studied.

MATERIALS AND METHODS

Culture and experimental methods

The rotifer used in this study was *Asplanchna brightwelli* Clone 4B61 originally obtained from Dr. John Gilbert, Dartmouth College, Hanover, NH. The rotifers were fed *Paramecium caudatum* cultured in a Cerophyll infusion medium and incubated with *E. coli* as described in a previous paper (Enesco and Verdone-Smith, 1980).

Rotifers were placed singly in the wells of Tissue Culture Clusters #3524 obtained from Costar, Cambridge, Mass. The wells were checked every 12 hours. The newborn rotifers were transferred into new culture chambers and divided into the various experimental groups.

Temperature experiments

Five groups of 24 rotifers were used for the temperature experiments. The groups were kept in the dark at 15°C, 17.5°C, 20°C, 23°C and 25°C in an incubator. The chambers were checked every 12 hours for deaths and for the presence of offspring, which were counted and discarded. In addition, 0.5 ml of medium was replaced to assure a constant, fresh supply of food for the rotifer.

Dietary restriction experiments

Six groups of 24 rotifers were used for this study and the various groups were fed by replenishing their medium with fresh paramecia-filled media at intervals of 12, 24, 36, 48, 60 and 72 hours. All experimental cultures were kept in the dark at 18°C. The chambers were checked every 12 hours. At the times when the rotifers were not fed, medium containing Cerophyll only was used to replenish the wells. The group fed every 12 hours may be considered as the full-fed control.

Statistical analysis

For both experiments, a one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey test was employed to determine whether there were any differences between the treatment groups in lifespan or fecundity.

RESULTS

Temperature experiment

The survival curves shown in Fig. 1 indicate that longevity in *A. brightwelli* was influenced by temperature. A one-way analysis of variance showed that there were significant differences among the mean lifespan of rotifers reared at the various temperatures ($F(4,115) = 17.87$, $p < .001$). The results show that there is a decrease in mean lifespan as temperature is increased. The longest mean lifespan occurred in the group reared at 17.5°C. *Post hoc* analysis revealed that this mean was not significantly different from the mean lifespan of rotifers kept at 15°C. The mean lifespan of *A. brightwelli* at these two temperatures was significantly longer than the mean lifespans of groups reared at 20°C, 23°C and 25°C. There were no significant differences in mean lifespan among the groups kept at the three higher temperatures. The mean lifespan in days for all the groups are given in Table 1.

Table 2 shows the reproductive patterns of *A. brightwelli* at the five temperatures. A one-way analysis of variance revealed significant differences in the time at which the rotifers entered the reproductive cycle ($F(4,115) = 14.35$, $p < .001$). The results demonstrate that the higher the temperature, the earlier the rotifers started reproducing. This relationship is emphasized in Fig. 2. The time at which the end of reproduction occurred was also significantly different at different temperatures ($F(4,115) = 56.42$, $p < .001$). Except for rotifers reared at 15°C, the results show that as lifespan increased at lower temperatures, the end of reproduction was delayed. The rotifers reared at 17.5°C dis-

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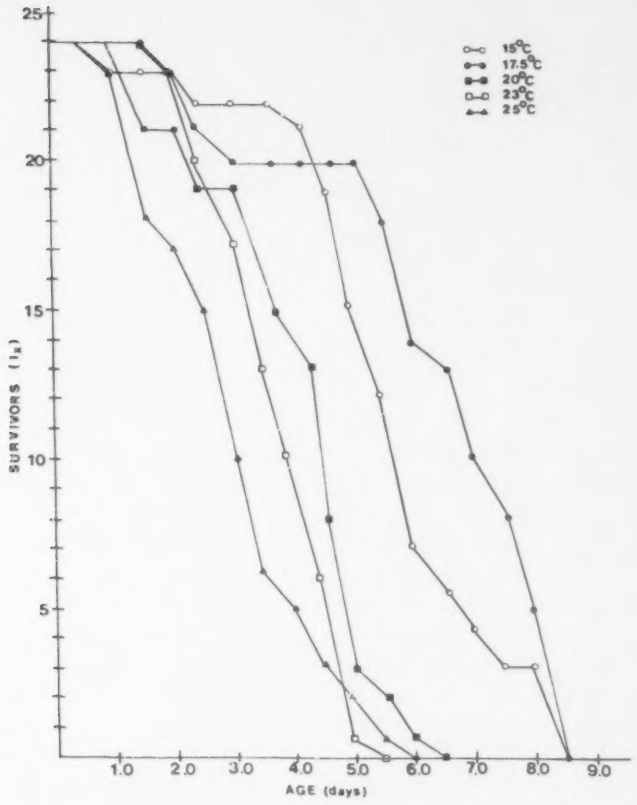

FIG. 1. Survival curves of *A. brightwelli* at five different environmental temperatures (N = 24 per group).

played a significantly longer reproductive period than any other group ($F(4,115) = 13.93$, $p < .001$). At this temperature, the average number of offspring produced per rotifer was also significantly higher than in any other group ($F(4,115) = 12.02$, $p < .001$). A more detailed presentation of the statistical analysis has been presented by Verdone-Smith (1981).

TABLE 1. MEAN LIFESPAN OF *Asplanchna brightwelli* AT VARIOUS ENVIRONMENTAL TEMPERATURES (N = 24). THE TEMPERATURE OPTIMUM WAS 17.5°C.

Temperature (°C)	Mean lifespan, (days) ± S.E.M.
15	5.62 ± .33
17.5	5.91 ± .41
20	3.93 ± .29
23	3.35 ± .20
25	3.04 ± .25

TABLE 2. REPRODUCTIVE PROFILE OF *Asplanchna brightwelli* REARED AT VARIOUS EXPERIMENTAL TEMPERATURES. (N = 24). FECUNDITY WAS GREATEST AT 17.5°C. R.P. INDICATES THE "REPRODUCTIVE PERIOD."

Temp. (°C)	Average start of R.P. (days) ± S.E.M.	Average end of R.P. (days) ± S.E.M.	Average length of R.P. (days) ± S.E.M.	Peak day of R.P.	Average number of offspring per rotifer
15	3.83 ± .31	4.85 ± .43	1.02 ± .28	4.5	2
17.5	2.20 ± .19	5.39 ± .47	3.19 ± .34	4.0	7
20	2.00 ± .25	3.37 ± .42	1.38 ± .23	3.5	4
23	1.81 ± .10	2.93 ± .19	1.15 ± .12	2.0	4
25	1.54 ± .26	2.37 ± .40	.83 ± .19	2.5	2

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Dietary restriction experiment

Fig. 3 shows the survival curves for the six groups of *A. brightwelli* fed at the various time intervals. A one-way analysis of variance showed that this form of dietary restriction significantly altered the mean lifespan of these rotifers ($F(5,138) = 6.29$, $p < .001$). The mean lifespan for all the groups is given in Table 3.

As the feeding interval was extended from 12 to 24 and to 36 hours, the mean lifespan increased slightly. *Post hoc* analysis revealed no significant differences among these three groups. More severe restriction was not beneficial to the survival of *A. brightwelli*. Rotifers fed at 48, 60 and 72-hour intervals had shorter mean lifespans. There were no significant differences among these groups. However, these groups all had significantly shorter lifespans than the groups fed every 12, 24 and 36 hours. The pattern obtained was thus a slight increase in longevity with moderate dietary restriction (up to a 36-hour feeding interval) followed by a decrease in survival with more severe restriction.

Table 4 shows the reproductive pattern for *A. brightwelli* during different intensities of dietary restriction. There were no significant differences in the times at which the rotifers entered the reproductive period under the various regimes ($F(5,138) = .891$, $p > .05$). Significant differences were found in the average time when the rotifers stopped reproducing ($F(5,138) = 6.25$, $p < .001$). The groups of rotifers fed at 12, 24 and 36 hour intervals stopped reproducing significantly later than the groups fed every 48, 60 and 72 hours.

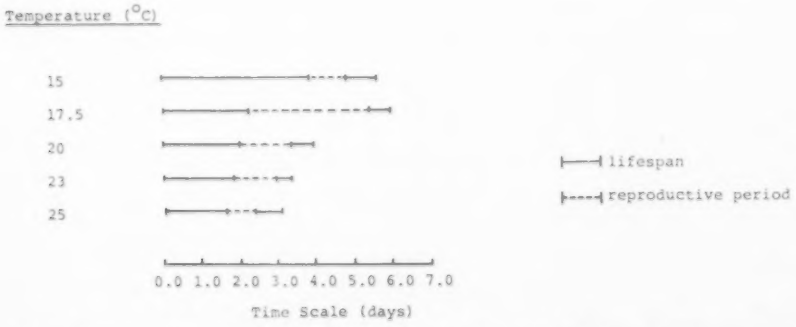

FIG. 2. Time occupied by the three phases of the life cycle of *A. brightwelli* at five different temperatures. Both the pre-reproductive and reproductive periods are influenced by temperature while the post-reproductive period remains constant.

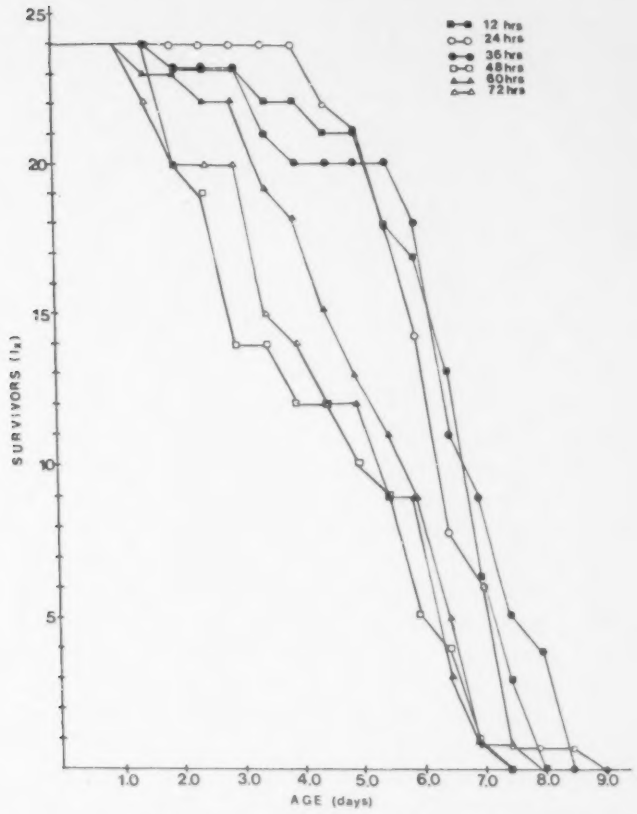

FIG. 3. Survival curves of *A. brightwelli* under various restricted diets (N = 24 per group). The group fed every 12 hours is considered the fully-fed control.

The mean length of the reproductive cycle was significantly longer in the three moderately restricted groups ($F(5,138) = 12.64$, $p < .001$). Rotifers that were severely restricted had increasingly shorter reproductive cycles correlated with the shorter lifespans. These results are illustrated in Fig. 4. As would be expected, the average number of offspring produced per rotifer was significantly reduced under severe dietary restriction ($F(5,138) = 24.65$, $p < .001$).

TABLE 3. MEAN LIFESPAN OF *Asplanchna brightwelli* AT 18°C ON VARIOUS RESTRICTED FEEDING INTERVALS (N = 24). LIFESPAN WAS LONGEST WHEN ROTIFERS WERE FED EVERY 36 HOURS.

Feeding interval (hr)	Mean lifespan, (days) ± S.E.M.
12	5.44 ± .28
24	5.91 ± .21
36	6.08 ± .34
48	4.41 ± .36
60	4.41 ± .32
72	4.35 ± .35

TABLE 4. REPRODUCTIVE PROFILE OF *Asplanchna brightwelli* AT 18°C UNDER VARIOUS RESTRICTED DIETS (N = 24 FOR EACH GROUP). R.P. INDICATES "REPRODUCTIVE PERIOD."

Feeding interval (hr)	Average start of R.P. (days) ± S.E.M.	Average end of R.P. (days) ± S.E.M.	Average length of R.P. (days) ± S.E.M.	Peak day of R.P.	Average number of offspring per rotifer
12	2.81 ± .14	5.29 ± .30	2.48 ± .21	3.5	7
24	2.95 ± .09	5.64 ± .21	2.69 ± .21	3.5	5
36	2.87 ± .20	5.64 ± .42	2.78 ± .28	2.5	6
48	2.29 ± .33	3.52 ± .55	1.27 ± .26	3.5	2
60	2.60 ± .33	3.60 ± .46	1.06 ± .21	4.0	2
72	2.47 ± .37	3.45 ± .54	.98 ± .23	4.0	2

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DISCUSSION

The results show that manipulating environmental factors such as temperature and diet markedly affected lifespan and reproduction in *A. brightwelli*.

One of the first theories that attempted to explain why temperature affected longevity in most organisms was proposed by Pearl in 1928 (cited in Lamb, 1977). His "rate of living" theory which extended from studies on *Drosophila* by Loeb and Northrop (1917) stated that lifespan was correlated with energy expenditure. It was observed that at high temperatures, poikilothermic organisms had higher metabolic rates, that the rates of development were increased and that the onset of sexual maturity occurred earlier; in addition, senescence and death occurred earlier when organisms were kept at higher temperatures. These observations confirmed an earlier hypothesis by Rubner (1908) who proposed that length of life was inversely proportional to the rate of metabolism (Strehler, 1977). Similar observations were reported in *Drosophila* (Loeb and Northrop, 1917; Miguel, Lundgeren, Bensch and Atlan, 1976), nematodes (Byerly, Cassada and Russell, 1976), rotifers (Meadow and Barrows, 1969) and various fishes and insects (Briegel and Kaiser, 1973; Van Heukelem, 1978).

Fanestil and Barrows (1965) have shown that *Philodina* reared at 25°C had a mean lifespan of 34 days which was significantly longer than those reared at 31°C and 35°C where

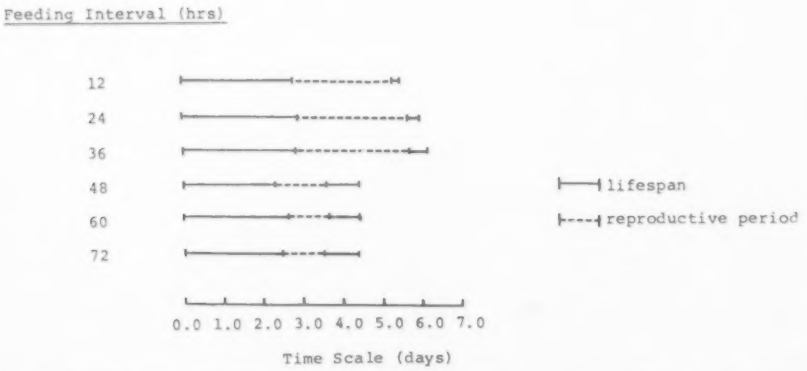

FIG. 4. Time occupied by the three phases of the life cycle of *A. brightwelli* under the various restricted diets. The reproductive period is influenced by the changes in feeding intervals while the pre-reproductive and the post-reproductive periods remain constant.

the mean lifespan was 22 days and 18 days respectively. The authors also reported that the total number of eggs produced by rotifers at 35°C was significantly reduced as compared to the other two groups. Meadow (1967) also reported that the lifespan of these rotifers was greater at 24°C than at 27°C. The study showed that although the number of eggs laid by the two groups of *Philodina* was similar, the egg-laying process began and ended earlier in the groups reared at 27°C.

In the present experiment using *A. brightwelli*, it was shown that the greatest mean lifespan of 5.91 days occurred at 17.5°C and that survival decreased as temperatures increased. It was also observed that the production of offspring started and ended earlier as temperatures increased except for rotifers reared at 15°C where reproduction stopped earlier than in rotifers at 17.5°C. Also, except for rotifers at 15°C, the length of the reproductive period decreased as temperatures increased. The average number of offspring produced per rotifer decreased as a function of the shorter reproductive periods brought about by the higher temperatures. Longevity was therefore increased in *A. brightwelli* by lower temperatures and the increase in mean lifespan was a function of longer pre-reproductive and reproductive periods. Higher temperatures seemed to cause the rotifers to mature and senesce faster. Thus, these results and those of other rotifer studies support the "rate of living" theory.

Dietary restriction was conducted by increasing the length of feeding intervals in the various groups of rotifers while maintaining them at 18°C. The mechanism by which dietary restriction acts to extend lifespan in organisms is unknown. Moment (1978) proposed that the answer may involve reduction in the use of the genetic code thus decreasing the wear and tear on DNA. Barrows and Kokkonen (1978) supported this argument by stating that protein synthesis was decreased through dietary restriction and that such a decrease in the use of the genetic code could minimize "genetic imperfections" arising in later life. Lamb (1977) suggested that dietary restriction slowed down the "biological clock" therefore slowing down the rate of senescence.

Although most dietary restriction experiments involved mammalian systems, a few studies were carried out using invertebrates. In *Tokophyra* it was shown that maximum lifespan was markedly increased from 10 to 80 days if the ciliates were fed twice daily instead of *ad libitum* or 7–8 times a day (MacKeen and Mitchell, 1975). Similarly, the lifespan of *Campanularia flexuosa* was significantly increased if fed *Artemia* every third day instead of every day; 50% survival occurred at 15 days in the fully fed group but at 23 days in the restricted group. (Brock, cited in Barrows and Kokkonen, 1978). Fanestil and Barrows (1965) have shown that longevity in *Philodina* was influenced by limiting the times of feeding and by the type of diet given. The authors reported that as mean lifespan was increased from 34 to 55 days through various restricted diets, the interval between the start and end of egg production also increased.

In *A. brightwelli*, the results presented here show that underfeeding by means of lengthening the feeding interval up to 36 hours increased the mean lifespan. Although the start of reproduction was similar in all the groups, the mean length of the reproductive period was increased as lifespan was increased through moderate dietary restriction. In the severely restricted groups, the reproductive cycle was shortened as lifespan decreased and as a result, the number of offspring produced in these groups was significantly reduced. It is interesting to note that the post-reproductive period was constant in all the groups. The end of the reproductive period could signal the onset of senescence very precisely in this species.

In *A. brightwelli*, moderate dietary restriction conducted in the manner described increased longevity. It would be possible to interpret the slightly lengthened lifespan in terms of the "rate of living" theory. Since the rotifers are consuming less food, they doubtless have a reduced energy expenditure. Although the onset of sexual maturity was not delayed, the period of reproduction was lengthened and senescence was delayed. It is of interest that "rate of living" may be influenced both by temperature and diet in this extremely short-lived species.

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Experimental Gerontology, Vol. 17, pp. 263–266, 1982
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MATERNAL AGE AND LIFESPAN DO NOT INFLUENCE LONGEVITY IN THE ROTIFER *ASPLANCHNA BRIGHTWELLI*

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(*Received* 8 *August* 1981)

Abstract—An analysis of the mean lifespans among the progeny of the viviparous rotifer *Asplanchna brightwelli* produced during four generations has revealed that longevity of the offspring is not affected by maternal age of the parent or by the parent's lifespan. The production of viable offspring at any time during the parent's reproductive period may be of importance in assuring the continuity of clones of this short-lived rotifer species.

INTRODUCTION

THE PURPOSE of this study was twofold: (1) To determine whether offspring of the viviparous rotifer *Asplanchna brightwelli* obtained at different times during the reproductive period of the "mother" rotifer showed differences in mean lifespan, and (2) To determine whether maternal lifespan had any influence on the lifespan of the offspring.

Several maternal age studies have been conducted using various species of egg-laying rotifers such as *Proales sordida*, Gosse (Jennings and Lynch, 1928), *Philodina citrina* (Lansing, 1947), *Philodina acuticornis* (Meadow and Barrows, 1971), *Brachionus calyciflorus*, Pallas (Rougier and Pourriot, 1977), and *Philodina roseola* (Ricci, 1980). The general conclusion from the early studies was that the lifespan of offspring varied inversely with maternal age; this phenomenon was attributed partly to a decrease in the viability of eggs produced by older females and possibly to changes in the external environment, mainly temperature, pH and nutrition. In contrast, the later studies revealed that maternal age had little influence on the lifespan of the progeny.

The form of *A. brightwelli* used in this study is parthenogenetic, therefore the offspring are released from the parent after they are fully developed. In this study we were interested in determining whether offspring born early vs. late in the parent's reproductive period displayed differences in mean lifespan or fecundity and whether the parent's lifespan influenced the lifespan of the offspring.

MATERIALS AND METHODS

Cultures of *Asplanchna brightwelli* clone 4B61 were originally obtained from Dr. John Gilbert, Dartmouth College, Hanover, NH. They were cultured on a cerophyll infusion medium incubated with *Paramecia cauda-*

tum and *E. coli* as described in a previous paper (Enesco and Verdone-Smith, 1980). The rotifers were kept singly in the wells of Tissue Culture Chambers (#3524) obtained from Costar, Cambridge, MA. The culture dishes were kept in the dark at 18°C and were fed paramecia every 12h. The chambers were checked every 12h for accurate determination of lifespan and number of offspring produced. An initial clone of 15 rotifers was isolated (G0) and records of mean lifespan and reproduction were kept for four successive generations (G1 to G4).

RESULTS

Table 1 shows the mean lifespan and reproductive profile for *A. brightwelli* in the initial generation (G0) and the four successive generations (G1 to G4). The data on reproduction includes the mean start and end of the reproductive period as well as the average number of offspring produced per rotifer in the various populations. The reproductive profile was calculated using only rotifers which produced offspring. In every population of rotifers used, about 30% of the sample are non-reproductive or die prior to entering the reproductive period. There were no significant differences among the mean lifespans of the five generations according to a one-way analysis of variance ($F[4,269] = 1.73$, $p \geq .05$). This statistical analysis was also carried out using only the rotifers which reproduced and again, there were no significant differences in the mean lifespans ($F[4,182] = .466$, $p > .05$)

The mean time at which the reproductive period began and ended was not significantly different among the five generations of rotifers ($F[4,182] = 1.01$; $F[4,182] = .922$, $p > .05$). In addition, the average number of offspring produced per rotifer in the five generations was not significantly different ($F[4,182] = 2.19$, $p > .05$).

In both sets of data presented, there were no indications of a transmissible or cumulative trend in mean lifespan from one generation to the other. The data were therefore pooled prior to the final statistical analysis.

Table 2 represents the mean lifespan of offspring born at different times during the parent's reproductive period. The range of time of the reproductive phase varied between a parental age of 4.0 to 6.0 days. The results of the mean lifespan for all four generations (G1 to G4) were pooled to obtain a larger sample size and these were recorded as G Total. A one-way analysis of variance showed that no significant differences occurred in the mean lifespan of the offspring whether these were born at an early or late maternal age ($F[4,221] = 1.430$, $p > .05$).

Table 3 shows the mean lifespan of offspring in relation to the total lifespan of its parent for four successive generations. A one-way analysis of the pooled data revealed no significant differences among the mean lifespans ($F[9,229] = .644$, $p > .05$). This indicated that the length of life of the parent did not influence the lifespan of its offspring.

TABLE 1. LIFESPAN AND REPRODUCTIVE PROFILE OF *A. brightwelli* FOR FIVE GENERATIONS (T = 18°C). AN *F*-TEST REVEALED NO SIGNIFICANT DIFFERENCES AMONG THE GROUPS.

Generation	*N*	Mean lifespan, *x* (days) ± SEM	*N* in *R.P.	Average start of R.P. (days)	Average end of R.P. (days)	Average number of offspring/ rotifer
G0	15	4.93 ± .62	10	4.15 ± .27	6.10 ± .56	3.4 ± .79
G1	44	5.59 ± .29	29	4.29 ± .35	5.91 ± .22	2.9 ± .31
G2	72	5.48 ± .27	49	4.34 ± .07	6.07 ± .21	3.3 ± .26
G3	72	6.15 ± .24	55	4.42 ± .10	6.45 ± .19	3.4 ± .25
G4	71	5.44 ± .23	44	4.14 ± .15	5.63 ± .25	2.6 ± .24

*R.P. = Reproductive Period.

TABLE 2. EFFECT OF MATERNAL AGE ON THE MEAN LIFESPAN OF OFFSPRING IN *A. brightwelli* FOR FOUR SUCCESSIVE GENERATIONS. (T = 18°C). AN *F*-TEST REVEALED NO SIGNIFICANT DIFFERENCES AMONG THE GROUPS.

Maternal age (days)	Mean lifespan of offspring (days)					
	G1	G2	G3	G4	*N*	G Total*
4.0	5.12	5.45	5.73	4.81	58	5.33 ± .25
4.5	5.75	5.00	6.00	6.37	64	5.72 ± .26
5.0	6.80	6.31	5.96	4.82	55	5.72 ± .25
5.5	7.37	4.95	7.58	6.64	27	6.33 ± .44
6.0	5.75	6.00	6.90	6.33	22	6.22 ± .43

*$\bar{x}$ ± S.E.M.

DISCUSSION

Several rotifer studies have been conducted to observe the effects of maternal age on the mean lifespan of offspring. The majority of these studies were carried out using species of egg-laying rotifers such as *Philodina* and *Proales sordida*.

Jennings & Lynch (1928) concluded from studies using *Proales sordida* that the lifespan of offspring varied inversely with maternal age. These authors found that as the female rotifer aged, there was an increase in the frequency of abortive or non-hatching eggs. Also, the progeny of old females showed a great variability in rates of development and fecundity. The eggs of old females varied greatly in shape and size which led the authors to suggest that individuals developing from smaller eggs were physiologically stressed as compared to offspring from larger eggs.

Lansing (1947) conducted a series of experiments using clones of young female (pediaclones) and old female (geriaclones) *Philodina citrina*. He observed that in the pediaclones, the mean lifespan increased in each successive generation while in the geriaclones, the mean lifespan decreased until extinction (after about 17 generations). This was due to both a decline in egg production and a decrease in the viability of eggs produced. A study by Meadow and Barrows (1971) using *Philodina acuticornis odiosa*, Milne, showed that there existed little difference in the mean lifespan between pediaclones and geriaclones. The authors found a significantly higher mean lifespan in the geriaclone only when the

TABLE 3. EFFECT OF MATERNAL LIFESPAN ON THE MEAN LIFESPAN OF OFFSPRING IN *A. brightwelli* FOR FOUR SUCCESSIVE GENERATIONS (T = 18°C). AN *F*-TEST REVEALED NO SIGNIFICANT DIFFERENCES AMONG THE GROUPS.

Total maternal lifespan (days)	Mean lifespan of offspring (days)					
	G1	G2	G3	G4	*N*	G Total*
5.0	–	5.42	6.25	6.00	15	5.80 ± .53
5.5	–	5.07	5.40	4.75	20	5.22 ± .55
6.0	–	4.21	6.65	5.50	26	5.59 ± .41
6.5	5.36	4.83	6.31	7.00	39	5.67 ± .35
7.0	–	5.80	6.16	5.43	42	5.69 ± .33
7.5	6.20	6.10	6.50	5.25	30	6.16 ± .31
8.0	5.91	4.75	–	3.37	14	4.85 ± .54
8.5	5.35	–	7.06	5.36	30	5.81 ± .35
9.0	–	–	7.50	5.38	12	5.91 ± .58
9.5	–	7.50	6.42	5.50	11	6.27 ± .45

*$\bar{x}$ ± S.E.M.

cultures were dixenic. They concluded that maternal age had no deleterious effects on the lifespan of the progeny but that the reproductive rate was slightly reduced in older females. A similar experiment was conducted by Ricci (1980) using the rotifer *Philodina roseola*; she found no differences in mean lifespan (or fecundity) among offspring of juvenile, mature and old rotifers but she did observe a wider range of variability in the eggs of the older females.

The results of the present experiment showed no significant differences in the mean lifespan of offspring that were born at various times during the parent's reproductive period; in addition, the parent's total lifespan had no influence on the length of life of the progeny. It was also observed that fecundity was not affected in rotifers born at different maternal ages. There were no significant differences in the number of offspring produced among the rotifers born at an early or late maternal age in any generation.

There are numerous differences between *A. brightwelli* and other rotifers used in similar studies, mainly: (1) *A. brightwelli* has a much shorter lifespan, (about five days) while the *Philodina* species display lifespans of over 25 days, and (2) *A. brightwelli* is a viviparous rotifer as opposed to an egg-laying type. As a function of these two characteristics, (3) the number of offspring is greatly reduced in *A. brightwelli* as compared to other rotifer species.

It is possible that the action of regulatory genes and physiological processes that promote egg-laying in some rotifers are more susceptible to the changes that occur with senescence. *Philodina*, for example, produces a great number of viable and non-hatching eggs throughout its long lifespan and since more abortive eggs occur in the older females, this may indicate the presence of deleterious genes as age increases. But the species survival is assured by the presence of many viable eggs in younger females.

In contrast, *A. brightwelli* has evolved other forms of reproductive strategy. This rotifer gives birth to a few, fully-developed offspring during its short lifespan and this may be a method of assuring the survival of the species. All offspring, whether born early or late during the reproductive period, are equally viable, thus assuring the continuity of the parthenogenetic clones. Another reproductive strategy in this species is the transformation from amictic to mictic reproduction under adverse conditions such as high temperatures or decreased food availability. This enables *A. brightwelli* to produce thick resting eggs which hatch in more favorable conditions and again survival of this rotifer species is assured (Gilbert, 1968).

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Experimental Gerontology, Vol. 17, pp. 267–272, 1982
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A QUANTITATIVE CYTOCHEMICAL STUDY OF ACID PHOSPHATASES IN HEPATOCYTES OF DIFFERENT PLOIDY CLASSES FROM AGING RATS

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Abstract—A quantitative cytochemical study of isolated hepatocytes from young, middle-aged and old adult rats has shown a doubling of acid naphthol AS-BI phosphatase activity as the hepatocytes shift from the diploid to the tetraploid state, and an overall increase in activity in cells from old animals. There was a complete inhibition of all acid phosphatase activity with 10^{-2}M sodium molybdate, but only a 90% inhibition in hepatocytes from livers of rats of 12–25 months-old animals with 10^{-2}M sodium fluoride, the resistant activity possibly representing adenyl cyclase activity. In contrast, 10^{-2}M ouabain inhibited 70–80% of the activity in hepatocytes from 12–15-months-old animals, this level of inhibition decreasing with increasing age, and implying a decrease in transport ATPase activity and an increased lysosomal phosphatase activity with increasing age of the rats.

INTRODUCTION

PREVIOUS QUANTITATIVE cytochemical studies of acid naphthol AS-BI phosphatase activities in isolated hepatocytes from young and middle-aged adult rats have shown (a) an increased activity of almost 100% when the euploid value of the liver cell doubles, and (b) that 70–80% of this activity is ouabain sensitive regardless of euploidy value (Middleton and Gahan, 1979).

The present work concerns an extension of these studies on isolated hepatocytes from rats of ages up to 32 months, together with the effects of various inhibitors on the acid phosphatases investigated.

MATERIALS AND METHODS

Materials

Adult female BN/BiRij rats aged from 12–32 months were obtained from the specific pathogen free colony maintained at the Institute for Experimental Gerontology, TNO, Rijswijk (Z.H.) in the Netherlands. These animals were maintained in small groups under constant temperature (21°C) and relative humidity conditions (60%). This strain of rat has a mean life span of 30.5 months and the advantage that neoplastic nodules and other lesions originating from hepatocytes are almost totally absent from old female BN/BiRij rats (Hollander, 1976).

Suspensions of isolated hepatocytes were dissociated from rat livers by use of collagenase (Sigma Type I) and hyaluronidase (Sigma Type I) (Van Bezooijen *et al.*, 1974; 1977). The isolated hepatocytes were maintained in

Dulbecco's medium plus 10% foetal calf serum. Over 90% of the cells used in the current experiments were viable as indicated by the trypan blue dye exclusion test (Grankvist *et al.*, 1977). The cells were prepared on microscope slides as previously described (Middleton and Gahan, 1979).

Quantification of acid phosphatase activity and DNA content of individual hepatocytes

Hepatocytes were incubated for acid phosphatase in a medium containing naphthol AS-BI phosphate as substrate and fast red violet LB as the diazonium salt at pH 5.4 for 75 min at 37°C. The enzyme reaction end-product for each cell was measured by its absorbance at 535 nm using a Vickers M86 integrating microdensitometer (Middleton and Gahan, 1979). Subsequently, the enzyme reaction end-product was removed from the cells by extraction in 40% n-heptanol in 1,1,2,2-tetrachloroethane (Maggi and Chng, 1966) after which the DNA was stained by means of the Feulgen reaction. The DNA values of the hepatocyte nuclei were measured by integrating microdensitometry at a wavelength of 540 nm. Rat sperm and white blood cell nuclei similarly treated served as standard material to establish haploid and diploid DNA values respectively.

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RESULTS

Acid phosphatase activities

Mean values for hepatocytes of different ploidy classes from rats of different ages are given in Table 1 alongside previously reported data for young adult rats (Middleton and Gahan, 1979) for comparison. The approximate doubling of activity previously seen on shifting from diploid cells (1 × 2c) to tetraploid cells (2 × 2c; 1 × 4c) is repeated in cells from 25 months and 32 months old animals. However, this is not so for the shift from tetraploidy to octoploidy when the increase in enzyme activity is only about 50% (Table 1).

Comparison of the activities from each age against 12½ months as 100% for each ploidy class shows a similar activity for 15m but a continuing increase in activity at 25 and 32 months, the activity doubling in the 1 × 2c and 1 × 4c values at 32 months (Table 1).

Inhibitors

Sodium molybdate (10^{-2}M) completely inhibited all activity in all hepatocytes from each age. However, sodium fluoride (10^{-2}M) only inhibited 90% of the activity in hepatocytes from rats of 12½-25 months. At 30 months, 99–100% of activity was inhibited by the fluoride ions.

The data for ouabain inhibition (Table 2) show that 70–80% inhibition occurs at 12½ and 15 months, declining slightly to about 65% at 25 months, and dropping to 38% or less by 32 months.

DISCUSSION

Comparison of the mean values of acid phosphatase activities in the isolated hepatocytes show a doubling in activity on shifting from diploid to tetraploid values in the 25-month animal and almost the same in the 32-month animals. This is as previously reported for younger adults (Middleton and Gahan, 1979). The main difference lies in the failure to double the activity from 4c to 8c in the older animals which may be interpreted as either an inability to double the activity, or the lack of need to double the activity in late age. Equally, such a doubling of activity need not be expected since a comparison of the results at 12–15 months and those of 25 and 32 months shows a trend towards a doubling in the total amount of activity for the 2c and 4c cells, and a 60–70% increase for the higher ploidy values (Table 1). Since the cell volumes appear to remain relatively constant within

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TABLE 1. MEAN ACID NAPHTHOL AS-BI PHOSPHATASE REACTION END-PRODUCT PER CELL ± S.D. FOR HEPATOCYTES OF VARIOUS CLASSES FROM RATS 12–32 MONTHS OLD. THE NUMBER OF CELLS MEASURED IS IN PARENTHESES.

	Age (months)	Ploidy class						
		1 × 2c	2 × 2c	1 × 4c	2 × 4c	1 × 8c	2 × 8c	1 × 16c
Mean reaction product (AIOD)** ± S D	12½*	61 ± 13 (10)	146 ± 37 (38)	126 ± 29 (214)	250 ± 49 (27)	266 ± 50 (3)	447 (1)	–
	15*	59 ± 12 (3)	127 ± 34 (20)	124 ± 25 (182)	227 ± 42 (30)	237 ± 53 (3)	–	–
	25	108 ± 26 (16)	234 ± 47 (23)	216 ± 37 (107)	386 ± 97 (31)	377 ± 80 (80)	569 ± 104 (12)	–
	32	124 ± 39 (24)	187 ± 66 (5)	214 ± 48 (69)	354 ± 61 (28)	299 ± 56 (103)	454 ± 89 (25)	394 ± 92 (5)
	32	127 ± 37 (15)	231 ± 63 (14)	251 ± 65 (107)	394 ± 86 (60)	377 ± 122 (57)	512 ± 109 (19)	564 ± 20 (3)
% Activity compared with 1×2c cells = 100%	12½*	100	239	207	410	436	733*	–
	15*	100	215	210	385	402	–	–
	25	100	217	200	357	349	527	–
	32	100	151	173	268	242	366	318
	32	100	182	198	310	297	403	444
% Activity compared with 12½m =100%	15*	97	87	98	91	89	–	–
	25	177	160	171	154	142	–	–
	32	203	128	170	133	112	–	–
	32	208	158	199	158	142	–	–

*From Middleton and Gahan (1979).

*Figure for 12½ month animal is from one cell only.

**AIOD = Absolute Integrated Optical Density.

TABLE 2. EFFECT OF OUABAIN INHIBITION.

	Age (months)	Ploidy classes					
		1 × 2c	2 × 2c	1 × 4c	2 × 4c	1 × 8c	2 × 8c
Mean	12½	13 ± 5 (7)	27 ± 5 (20)	25 ± 6 (94)	50 ± 10 (18)	58 (1)	–
reaction	15	17 ± 7 (8)	37 ± 13 (21)	37 ± 12 (118)	70 ± 22 (29)	80 ± 37 (4)	–
product	25	38 ± 10 (11)	84 ± 12 (15)	72 ± 15 (62)	136 ± 32 (17)	138 ± 27 (53)	187 ± 48 (8)
(AIOD)*	32	78 ± 30 (9)	162 ± 62 (2)	160 ± 35 (12)	254 ± 52 (17)	244 ± 112 (11)	423 ± 120 (4)
± S. D.	32	88 ± 24 (13)	144 ± 41 (8)	166 ± 56 (98)	258 ± 59 (42)	251 ± 90 (34)	405 ± 143 (15)
% inhibition	12½	79	81	80	80	78	–
v test	15	71	71	70	69	66	–
(Table 1)	25	65	64	67	65	63	67
	32	37	23	25	28	18	7
	32	31	38	34	34	33	21

*AIOD = Absolute Integrated Optical Density.

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each ploidy class regardless of age, then this may be interpreted as a real increase in acid phosphatase activity of the cells from the older animals. These data are in agreement with previously published biochemical data from hepatocytes of the same strain of rats (Sleyster and Knook, 1980).

Naphthol AS-BI phosphate is a broad-spectrum substrate (Barka, 1961; Gahan *et al.*, 1978; Meijer *et al.*, 1980) and will demonstrate at least three liver cell acid phosphatases. Total inhibition in the presence of molybdate ions has confirmed the activity of acid phosphatase. Furthermore, 90% inhibition was achieved with sodium fluoride in cells from 12–25-months animals and total inhibition at 32 months also indicating the presence of acid phosphatase. However, a small fluoride-resistant fraction which is absent from 32 months material has been noted in a variety of tissues (Maggi *et al.*, 1966; Maggi and Carbonell, 1969; Gahan *et al.*, 1978) and may be indicative of adenyl cyclase activity in the hepatocytes.

Some 70–80% inhibition is achieved with ouabain in hepatocytes from 12½–15-month rats, though with increasing age of the rat there is a decrease in the amount of activity inhibited (Table 2). The degree of inhibition is similar for all ploidy classes of a given age group. These results enable a distinction to be made between the classical lysosomal acid phosphatase which is ouabain resistant and sensitive to fluoride and molybdate, and the ouabain-sensitive fraction which may represent an ATPase fraction. The former fraction increases in activity in total amount in a small way until 25 months and then sharply by 32 months. The latter responds in the converse fashion, namely, decreases slowly to 25 months and drops sharply by 32 months. The ouabain-sensitive acid phosphatase is not restricted to the cell membrane but is seen also to be associated with cytoplasmic granules. Electron microscope cytochemical studies of acid naphthol AS-BI phosphatases in mouse kidney and seminal vesicle and hamster cardiac muscle have shown them to be associated with lysosomes, golgi stacks and endoplasmic reticulum (Maggi, 1973). Hence if this ouabain-sensitive fraction represents a transport phosphatase, at least part of this may be associated with the lysosomal system.

The enzyme substrate employed in these studies may be considered as demonstrating an increased lysosomal phosphatase activity with increased age, and a transport phosphatase which is diminished in activity in old age. These findings also confirm the need to study genuinely old adults in addition to those from late middle age in order to ascertain real changes related to aging.

Acknowledgements—We wish to thank Dr. D.L. Knook for supplying the liver material and for facilities in his laboratory for part of this work. We are indebted for financial assistance to the Science Research Council, U.K. (to P.B.G.) and to the E.E.C. Special Concerted Action Group on Cell Ageing and Organ Function.

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Experimental Gerontology, Vol. 17, pp. 273–279, 1982
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0531-5565/82/040273-07$03.00/0
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LEVELS OF LIPID PEROXIDATION IN HEPATOCYTES ISOLATED FROM AGING RATS FED AN ANTIOXIDANT-FREE DIET

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(*Received* 4 *September* 1981)

INTRODUCTION

ONE THEORY of cellular aging proposed initially by Harman (1956) is the process of lipid peroxidation. The lipid peroxidation concept states that free radicals from either endogeneous or exogeneous sources react with oxygen to attack and damage lipids in the membranes of the cell (for reviews see Leibovitz and Siegel, 1980; Gordon, 1974). Elevated lipid peroxidation in aging animals has been demonstrated in various tissues including the lungs (Kehrer and Autor, 1977), the RBC (Walls *et al.*, 1976), the brain (Sharma, 1977) and the liver (Player *et al.*, 1977). An important aspect of this theory is the role of antioxidants such as vitamin E. Because of their capacity to inhibit lipid peroxidation, antioxidants or free radical scavengers have been suggested to enhance longevity (Harman, 1968).

Although free radicals can be generated by exogeneous agents such as CCl_4 (Recknagel *et al.*, 1977), there is also a great deal of evidence that low levels of naturally occurring endogeneous free radicals are generated from cellular metabolism (McCay, 1981). The cell has several inherent defense systems for quenching these free radicals, for example enzyme systems like superoxide dismutase or glutathione peroxidase. Recently Hogberg (1977) utilized the enzymatically isolated hepatocyte as a research tool to study the cellular defenses against lipid peroxidation. Furthermore, several investigators (Britton *et al.*, 1976; Van Bezooijen, 1978) have utilized the isolated hepatocyte in the study of cellular aging. This paper describes the use of isolated rat hepatocytes to further investigate the role of lipid peroxidation in liver cell aging. Initially, the contribution of exogeneous and endogeneous factors to the level of lipid peroxidation in aging rat hepatocytes was assessed. Subsequently, rats were placed on an antioxidant-free diet for three weeks in an attempt to evaluate the free radical quenching or peroxidation protection capabilities of hepatocytes from young, adult and aged animals.

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MATERIALS AND METHODS

Fisher-344 female albino rats obtained from the National Institute of Aging's barrier-protected pathogen free colony at Charles River, (Wilmington, MA) were used throughout. When received, all animals were housed for three weeks at constant temperature and humidity, maintained on a 12/12 light-dark cycle, and housed in barrier-protected cages. The animals were pair-fed either sterilized Lab Chow and water or an antioxidant-free liquid diet (#6110E Lieber-DiCarli Liquid Rat Diet from Bio-Serv Inc., Frenchtown, NJ) for three weeks. Body weight was measured every other day, while diet consumption was measured daily.

Hepatic parenchymal cells were isolated from rats fasted overnight based on the isolation procedure outlined by Wagle and Ingebretsen (1975) with the following modifications. Livers from rats anesthetized with ether were initially perfused for 10 minutes with calcium-free bicarbonate buffered Hanks Balanced Salt Solution (BSS). The liver was subsequently perfused for 10 minutes with Hanks BSS with calcium and collagenase (35 mg%, Type II, Worthington Biochemical Corp.). Cell counts were made with a hemocytometer and cell viability ascertained by trypan blue dye exclusion (Hanks and Wallace, 1958).

Isolated cells were incubated in L-15 medium without phenol red modified with 150 mg/l of L-Tyrosine (ph 7.4, MA Bioproducts, Walkersville, MD) and 2% albumin (Type V, Sigma, St. Louis, MO). The L-15 medium was chosen for its capacity to maintain glutathione levels in isolated hepatocytes (Vina *et al.*, 1978). Cells were incubated at 37°C on a Dubnoff metabolic shaker in siliconized 25 ml Erlenmeyer flasks gassed at the start with 95% 0_2 and 5% CO_2. In all experiments the final volume per flask was 4–5 ml ($15–20 \times 10^6$ cells/ml). Cumene hydroperoxide (a,a-dimethylbenzyl hydroperoxide) as an exogeneous source of lipid peroxidation was obtained from MCB (Norwood, OH). Reduced nicotinamide adenine dinucleotide phosphate (NADPH, 0.4 mM) as a source of endogeneous lipid peroxidation was obtained from Sigma. Initially ADP (1.0 mM, Sigma) was chelated with Fe Cl_3 (.05 mM). This ADP-FE^{+3} complex was added with NADPH in all experiments.

Levels of lipid peroxidation in the isolated hepatocytes were determined by measuring malonaldehyde, a byproduct of the peroxidation process. The assay used was that of Nakamura and Hishinuma (1978). Two ml. of cells were initially solubilized with 0.4 ml. of a 7% lauryl sulfate solution; two ml. of 0.1 N HCl and 0.6 ml. of 10% phosphotungstic acid were then added. After addition of 1 ml of 0.63% thiobarbituric acid, the solution was heated for 60 minutes at 95°C. After cooling, 5 ml of n-butanol were added, the resulting solution shaken vigorously and centrifuged at 1000xg for 10 minutes. The resulting pink color in the n-butanol layer was measured colorimetrically at 535 nm with a spectrophotometer. A malonaldehyde (MDA) standard was prepared from the hydrolysis of 1,1,3,3-tetramethoxypropane according to Sinnhuber and Yu (1958). Values are expressed as micrograms (μg) of malonaldehyde/10^6 cells.

Data are expressed as the mean ± one standard error. Group means were compared using a two-way analysis of variance and the Student Newman-Keuls Multiple Range test at the 95% level of confidence.

RESULTS

Both daily diet (calorie) consumption and total body weight gain were similar in all groups of rats maintained for 21 days on the antioxidant-free diets (Table 1). However, the aged animals appeared to be more affected by the diet than the young or adult animals. Their eyes were often encrusted, their hair was falling out and they appeared listless. All

TABLE 1. DAILY DIET CONSUMPTION AND TOTAL BODY WEIGHT GAIN OF RATS FED AN ANTIOXIDANT-FREE DIET FOR THREE WEEKS.*

Age	*N*	Diet consumed (cal/day)	Total weight gain (g)
Young (2 months)	13	81 ± 2	31 ± 2
Adult (12 months)	12	85 ± 1	27 ± 1
Aged (24 months)	10	85 ± 2	28 ± 2

*Values represent mean ± one standard error. Fisher-344 female rats were obtained from the NIA Colony at Charles River (Wilmington, MA). Antioxidant-free liquid diet was obtained from Bio-Serv Inc. (Frenchtown, NJ).

isolated hepatocyte preparations showed an initial cell viability of greater than 80% with an average cell yield of 200 × 10^6 per animal. Both initial cell viability and cell yield were not significantly different either between age groups (young, adult or aged) or between diet groups (Lab Chow or the antioxidant-free diet).

Lipid peroxidation was assessed in isolated hepatocytes by measuring levels of malonaldehyde. With the Lab Chow diet levels of lipid peroxidation produced in isolated hepatocytes following 60 minutes' incubation were greatest in cells isolated from young (3-months-old) animals (Table 2). Hepatocytes from aged (25-months-old) animals maintained on a Lab Chow diet had the lowest of lipid peroxidation. Levels of lipid peroxidation following incubation in hepatocytes isolated from animals fed an antioxidant-free diet for 21 days were all significantly elevated when compared to comparable age groups fed Lab Chow (Table 2). Cells from young animals on the antioxidant-free diet showed a 2.8-fold increase in lipid peroxidation (6.0 to 16.8 μg of malonaldehyde per 10^6 cells) over cells isolated from young animals fed Lab Chow. Cells isolated from adult animals on the antioxidant-free diet showed a 2.3-fold increase in lipid peroxidation over cells isolated from adult animals fed Lab Chow (from 4.6 to 10.7 μg of malonaldehyde per 10^6 cells). Aged animals showed the largest increase in lipid peroxidation. Hepatocytes isolated from aged animals maintained on an antioxidant-free diet showed a 3.5-fold increase over hepatocytes isolated from aged animals maintained on Lab Chow (an increase from 3.3 to 11.7 μg of malonaldehyde per 10^6 cells).

In an effort to evaluate the peroxidation defense systems in hepatocytes isolated from animals fed the antioxidant-free diet, cumene hydroperoxide or reduced nicotinamide adenine dinucleotide phosphate (NADPH) was added to the incubation flasks to induce lipid peroxidation (Table 3). Cumene serves as an exogenous non-enzymatic source of lipid peroxidation (Hogberg, 1977). NADPH, on the other hand, serves as an endogeneous enzymatic source of lipid peroxidation (McCay *et al.*, 1981). All cells in the presence of either cumene or NADPH increased their malonaldehyde production when compared

TABLE 2. LIPID PEROXIDATION IN HEPATOCYTES ISOLATED FROM RATS FED LAB CHOW OR ANTIOXIDANT-FREE DIETS.*

Age	Malonaldehyde production (μg/10^6) cells)	
	Lab Chow	Antioxidant-free
Young (2 months)	6.0 ± 0.3 (15)**	16.8 ± 1.1**† (19)
Adult (12 months)	4.6 ± 0.2 (10)	10.7 ± 0.8† (19)
Aged (24 months)	3.3 ± 0.2 (13)**	11.7 ± 0.9† (19)

*Values represent the mean ± one standard error with the number of cell isolations in parentheses. Malonaldehyde was measured as an index of lipid peroxidation utilizing the thiobarbituric acid assay. Cells were incubated for 60 minutes in siliconized Erlenmeyer flasks in L-15 medium supplemented with 2% bovine albumin (15–20 × 10^6 cells/ml). Cells were derived from Fisher-344 female rats obtained from the NIA Colony at Charles River (Wilmington, MA). Animals were maintained on Lab Chow or antioxidant-free liquid diets (Bio-Serv, Frenchtown, NJ) for three weeks prior to cell isolations.

**Indicates statistical significance of $p < .05$ within the diet group.

†Indicates statistical significance of $p < .05$ between diets for comparable age groups.

TABLE 3. LIPID PEROXIDATION IN ISOLATED HEPATOCYTES IN THE PRESENCE OF CUMENE HYDROPEROXIDE OR NADPH.*

Age	N	Malonaldehyde production ($\mu g/10^6$ cells)		
		Control	Cumene hydroperoxide (0.2mM)	NADPH (0.4mM)
Young (2 months)	19	16.8 ± 1.1**†	21.7 ± 1.1**†	32.1 ± 1.5**†
Adult (12 months)	19	10.7 ± 0.8	13.1 ± 0.6	22.4 ± 1.2
Aged (24 months)	19	11.7 ± 1.0**	16.3 ± 0.9**	25.8 ± 1.3**

*Values represent the mean ± one standard error. Malonaldehyde was measured as an index of lipid peroxidation utilizing the thiobarbituric acid assay. Cells were incubated for sixty minutes in siliconized Erlenmeyer flasks in L-15 medium supplemented with 2% bovine albumin (15-20 × 10^6 cells/ml). Cells were derived from Fisher-344 female rats obtained from the NIA colony at Charles River (Wilmington, MA). All animals were maintained for three weeks prior to cell isolation on antioxidant-free liquid diets (Bio-Serv Inc., Frenchtown, NJ).

**Indicates significant differences from comparable adult values, $p < .05$.

†Indicates significant differences from comparable aged values, $p < .05$.

to control values (Table 3). Hepatocytes isolated from young animals maintained on the antioxidant-free diet had significant elevations in levels of lipid peroxidation when compared to either adult or aged cells. This was true both with the control values as well as in the presence of cumene or NADPH (Table 3). Although the levels of lipid peroxidation were not significantly elevated in the aged control cells, the aged hepatocytes did demonstrate significant elevations over adult hepatocytes when incubated with either cumene or NADPH (Table 3).

Cell viability via trypan blue dye exclusion was also assessed in hepatocytes isolated from animals maintained on the antioxidant-free diet (Table 4). Viability of isolated hepatocytes following incubation was not significantly different in the control flasks of either young, adult or aged cells (Table 4). Cumene clearly reduced viability in all groups, but most profoundly in the young hepatocytes (Table 4). These values also correspond to concurrent elevations in lipid peroxidation (see Table 3). NADPH also significantly reduced viability in all groups when compared to control values (Table 4). However, NADPH generated no significant difference in viability between the young, adult or aged hepatocytes (Table 4). Interestingly, NADPH induced the highest levels of lipid peroxidation in young, adult and aged hepatocytes (Table 3), yet the effect of NADPH on viability was not nearly so profound as that of cumene.

DISCUSSION

Young, adult and aged rats fed the antioxidant-free diet gained weight equally throughout the experiment (Table 1). The aged animals, however, appeared to be affected by the diet. They demonstrated alopecia, their eyes often became encrusted and they appeared listless.

The antioxidant-free diet clearly enhanced lipid peroxidation in isolated hepatocytes in all age groups when compared to cells isolated from rats maintained on Lab Chow (Table 2).

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TABLE 4. VIABILITY OF ISOLATED HEPATOCYTES IN THE PRESENCE OF CUMENE HYDROPEROXIDE OR NADPH.*

Age of rat	N	Percent viable		
		Control	Cumene hydroperoxide (0.2mM)	NADPH (0.4mM)
Young (2 months)	11	79 ± 2	23 ± 4**	66 ± 2
Adult (12 months)	11	73 ± 1	53 ± 4	66 ± 2
Aged (24 months)	11	80 ± 1	52 ± 3	69 ± 1

*Values represent the mean ± one standard error. All animals were Fisher-344 female rats derived from NIA Colony at Charles River (Wilmington, MA). Cell viability was determined via the trypan blue dye exclusion assay. Initial (zero time) viability for all cell isolations was greater than 80% and did not differ significantly between age groups. Cells were incubated for 60 minutes in siliconized Erlenmeyer flasks in L-15 medium supplemented with 2% bovine albumin (15–20 × 10^6 cells/ml). All animals were maintained on antioxidant-free liquid diets (Bio-Serv Inc., Frenchtown, NJ) three weeks prior to cell isolations.

**Indicates statistical difference of $p < .05$ from adult and aged values.

Hepatocytes isolated from young rats had the highest levels of malonaldehyde production (Table 2). This was true whether the animals were fed Lab Chow or the antioxidant-free diet (Table 2) as well as whether the cells were incubated in the presence of cumene or NADPH (Table 3). Since malonaldehyde is both produced and metabolized by the liver cell, this increase in the young cells may represent a difference in levels of metabolism compared to adult liver cells rather than an increase in production levels. A further expression of the differences in the young hepatocytes is found in the levels of cell viability. Following incubation with cumene the viability (via the trypan blue assay) of young hepatocytes was one-half that of the adult or aged hepatocytes (Table 4). Young animals have shown differences in drug metabolism and enzyme inducibility (Britton *et al.*, 1976) when compared to adults. The younger hepatocytes may not have yet developed an effective peroxidation defense system. Previous research, however, found lower levels of lipid peroxidation in young animals (Tappel, 1973; Sagai and Ichinose, 1980). However, these results were based on tissue homogenates of whole organs whereas the results in this paper are based on intact isolated hepatocytes. The differences, therefore, may be due to inhibitors of the peroxidation process found within the total organ (i.e., hormones) but not present in the isolated cell preparation. Finally, the stress of cell isolation may be greater for the younger hepatocytes than for the adult or aged cells thus resulting in greater levels of lipid peroxidation.

Isolated hepatocytes from aged rats fed Lab Chow demonstrated low levels of lipid peroxidation. This differs from previous results, which demonstrated increases in lipid peroxidation in aged animals (Tappel, 1973; Sagai and Ichinose, 1980). However, these results were based on whole tissue homogenates or the total organism. Our results imply that previously recorded increases in lipid peroxidation with age may be generated by extra-cellular sources (i.e., hormones, drugs). Supporting this concept are results by Van Bezooijen (1978) with isolated hepatocytes demonstrating that aged hepatocytes both structurally and functionally are very similar to adult hepatocytes.

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However, the aged liver cells do appear to have deficient lipid peroxidation defenses. Increases in levels of lipid peroxidation induced by the antioxidant-free diet were greatest (3.5-fold) in aged hepatocytes (Table 2). Furthermore, significant increases in lipid peroxidation were induced in the aged hepatocytes by both cumene and NADPH when compared to adult values (Table 3). The aging liver cell appears to be susceptible to the lipid peroxidation process due to a defective defense mechanism. Interestingly, Reiss and Gershon (1976) demonstrated that levels of superoxide dismutase decrease with advancing age in the rat. Furthermore, several researchers have implicated the superoxide anion in the peroxidation process. Barrett and Horton (1975) found no changes in lipid peroxidation levels in freshly isolated mitochondria from rats of various ages. However, when peroxidation was induced in incubated mitochondria significant increases with age developed. They postulated that these increases in induced peroxidation with age may be due to increases in proportions of polyunsaturated fatty acids with age.

It is also of interest to note that viability as measured by trypan blue did not appear to correlate with levels of induced lipid peroxidation (Tables 3 and 4). Although NADPH generated the greatest levels of lipid peroxidation (Table 3), its effect on viability was not nearly so profound as cumene (Table 4). One explanation for this might be the nature of the trypan blue test. Although the test does distinguish dead or severely damaged cells, it may not be sensitive enough to distinguish cells with mild injuries. Furthermore, these results might indicate differences in induced lipid peroxidation. Exogeneous agents such as cumene or CCl_4 induce non-enzymatic lipid peroxidation which may be more injurious than enzymatic lipid peroxidation induced via NADPH. Finally, it might be postulated that cell viability and lipid peroxidation (via trypan blue) may not be closely related. This concept has recently been proposed by Stacey and Klaassen (1981).

The results of this paper suggest that hepatocytes isolated from young and aged rats are vulnerable to the lipid peroxidation process. Aged hepatocytes demonstrated elevations in lipid peroxidation following 21 days of an antioxidant-free diet as well as following stress from cumene hydroperoxide or NADPH. Changes in levels of peroxidation found in the aged hepatocytes appear to be due to a decline in their defense against the peroxidation process.

SUMMARY

Enzymatically isolated hepatocytes were utilized to evaluate levels of lipid peroxidation in young (3 months), adult (12 months) and aged (25 months) Fisher-344 female rats. Lipid peroxidation was measured by assaying levels of malonaldehyde, a by-product of the peroxidation reaction. Young, adult and aged animals were fed a liquid antioxidant-free diet for 21 days prior to the hepatocyte isolation. Cells isolated from young rats demonstrated the highest levels of lipid peroxidation (μg of malonaldehyde/10^6 cells). This increase may be a result of the isolation procedure or of metabolic differences found in younger animals. When hepatocytes from aged animals on the antioxidant-free diet were incubated in the presence of lipid peroxidation inducers, such as cumene hydroperoxide or NADPH, significant elevations in lipid peroxidation over comparable adult values were observed. Aged hepatocytes appear to be more susceptible to the peroxidation process than adult hepatocytes, possibly due to defective peroxidation defenses.

Acknowledgement – This research was supported by NIH grant AG01407 from the National Institute on Aging.

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Experimental Gerontology, Vol. 17, pp. 281–286, 1982
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FUNCTIONAL PROPERTIES OF RAT LIVER PROTEIN SYNTHESIZING MACHINERY IN RELATION TO AGING

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(*Received* 18 *November* 1981)

Abstract—It is well established (van Bezooijen *et al.*, 1977a) that the protein synthesis by isolated liver paranchymal cells (ILPC) from Wag/Rij rats is dependent in a characteristic way on the age of the donor. Whereas cells of middle-aged animals exhibit a decrease in proteo-synthesis activity under *in vitro* incubation conditions as compared to cells of young rats, a marked increase is observed between 24 and 36 months of age. To investigate whether this overall age-related variation is directly correlated with an intrinsic change at the level of the protein synthesis apparatus of hepatic cells, we compared the size distribution and the in vitro translational activity of polysomes from four age groups. We show that both exhibit the same biphasic response to aging as does the protein synthesizing capacity of ILPC.

INTRODUCTION

WHEN TRYING to unravel the mechanisms of aging, we are tempted to believe that they are somehow controlled by the genetic apparatus. We therefore expect aging to induce changes at the level of the expression of genetic information. So, it is not surprising that a great amount of data has been published on the effects of aging on the rate of protein synthesis. (For a recent review, see Richardson, 1981.) Among the numerous investigators using rodent liver, most agree that the protein synthesis activity decreases during senescence. Two groups of investigators, however, have reported that cell-free protein synthesis does not change with age (Chen *et al.*, 1973; Beauchene *et al.*, 1967). It was reported by van Bezooijen (1977a) that the protein synthesizing capacity of ILPC from Wag/Rij rats decreases between 3 months and 12 months while there is an increase after 24 months.

This finding may appear intriguing at first sight and could possibly explain the discrepancies observed in the literature. These results have been confirmed by Coniglio *et al.* (1979) using ILPC from Fisher rats and this prompted us to investigate the functional state of the protein synthesizing apparatus of hepatocytes as a function of age. Since the sedimentation profile of polysomes, particulary the proportion of vacant ribosomes, depends on the ratio of the initiation rate versus the elongation rate (Palmiter, 1975) one may consider this parameter as a gross index of the functional state of the protein synthesis machinery. However, to be able to interpret changes in sedimentation profiles in

terms of a changed output of proteins one must also measure the *in vitro* translational capacity of polysomes.

MATERIALS AND METHODS

Preparation of polysomes and analyses of their sedimentation profile

Total polysomes from liver fragments of female Wag/Rij rats were prepared according to Ramsey and Steele (1976). After resuspending polysomes in bidistilled water, they were layered on to 15–50% (w/w) sucrose gradients made up in Tris-HCl 10 mM, pH 7.4, KCl 75 mM, Mg acetate 5 mM, EDTA 0.5 mM and DTT 3 mM, and centrifuged at 60.000 rpm (Beckman SW 60) for 50 minutes. The gradients were analyzed by pumping the contents through a flow cell in a recording spectrophotometer (ISCO, UA5) to yield a continuous trace of the absorbance profile at 254 nm. Sedimentation profiles were quantified by planimetry.

Assay of the in vitro *translational capacity*

20 μg of rat liver ribosomal fraction were incubated in a 25 μl assay with the components of the NEN ^{3}[H]-leucine translation kit. All components were used as recommended by the manufacturer except that the nuclease-treated reticulocyte lysate was centrifuged for 1 hour at 100.000 g (Beckman airfuge) prior to use in order to pellet the ribosomes. Incubations were performed at 25°C and the radioactivity incorporated into hot TCA-insoluble material was determined at different time intervals in 2 μl aliquots.

RESULTS AND DISCUSSION

The size distribution of hepatic polysomes from 3, 12, 24 and 36 months old rats were compared. Fig. 1 which represents a typical experiment, shows that a marked increase in

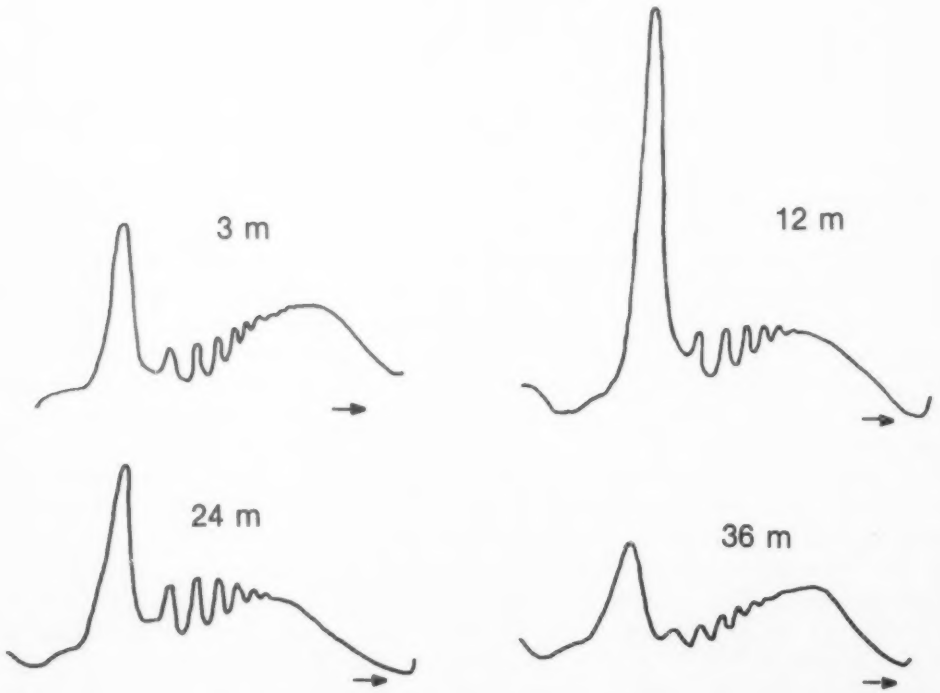

FIG. 1. Sedimentation analysis of hepatic polysomes of 3, 12, 24, and 36 months-old female Wag/ Rij rats (one typical experiment). Sedimentation analysis of polysomes was performed as described in Materials and Methods. One typical experiment is represented.

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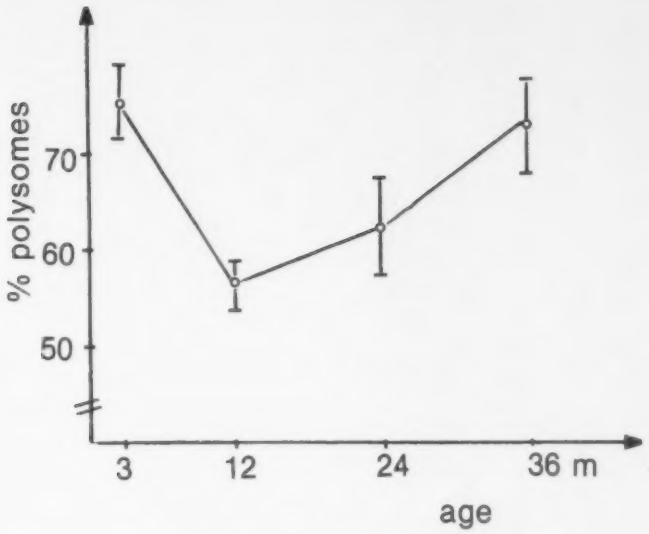

FIG. 2. Proportion of active ribosomes/total ribosomes as a function of age. Sedimentation profiles of hepatic polysomes of Wag/Rij rats of different age groups were quantified by planimetry. In order to determine the proportion of active ribosomes the area under the polyribosome peaks ($n \geq 2$) was divided by the total area under the profile; for the four age groups this value was respectively: 76.0 ± 4.2 (5), 56.8 ± 1.5 (3), 62.4 ± 5.0 (4) and 72.3 ± 6.0 (3) (mean ± S.D. the number of experiments between parentheses).

the proportion of monosomes occurs between 3 m and 12 m. The 24 m profile is not significantly different from the 12 m one while the percentage of monosomes decreases again between 24 m and 36 m. This finding is reproducible; the results of several experiments were quantified by planimetric analysis of the sedimentation profiles (Fig. 2).

In a second set of experiments, we incubated total ribosomal fractions from rat livers of different ages in a ribosome-free micrococcal nuclease-treated lysate and determined the incorporation of ^{3}H-leucine into hot TCA-insoluble material as a function of time. Under our conditions, the incorporation reaction is not far from steady state for about 30 min. after which it slows down and a plateau level is reached at about 60 min. The comparison of the kinetics for ribosomal fractions of different ages reveals that both the initial incorporation rate and the plateau level decrease between 3 m and 12 m. Such an age-related decrease in protein synthesis (which has been reported by many authors) has been claimed to be involved in the mechanism of aging. Previous results from this laboratory in mouse liver appeared to be consistent with the idea that a reduced recycling capacity of ribosomes is responsible for this decrease (Vandenhaute *et al.*, 1980.). We also observed a slight resurgence in the translation capacity between 12 m and 24 m and a significant one between 24 and 36 m (Fig. 3 and 4).

Considering sedimentation profiles and leucine incorporation kinetics as functions of age, we note that they both exhibit the same biphasic response to aging as does the protein synthesizing capacity of ILPC as determined by van Bezooijen (1977a). The possible

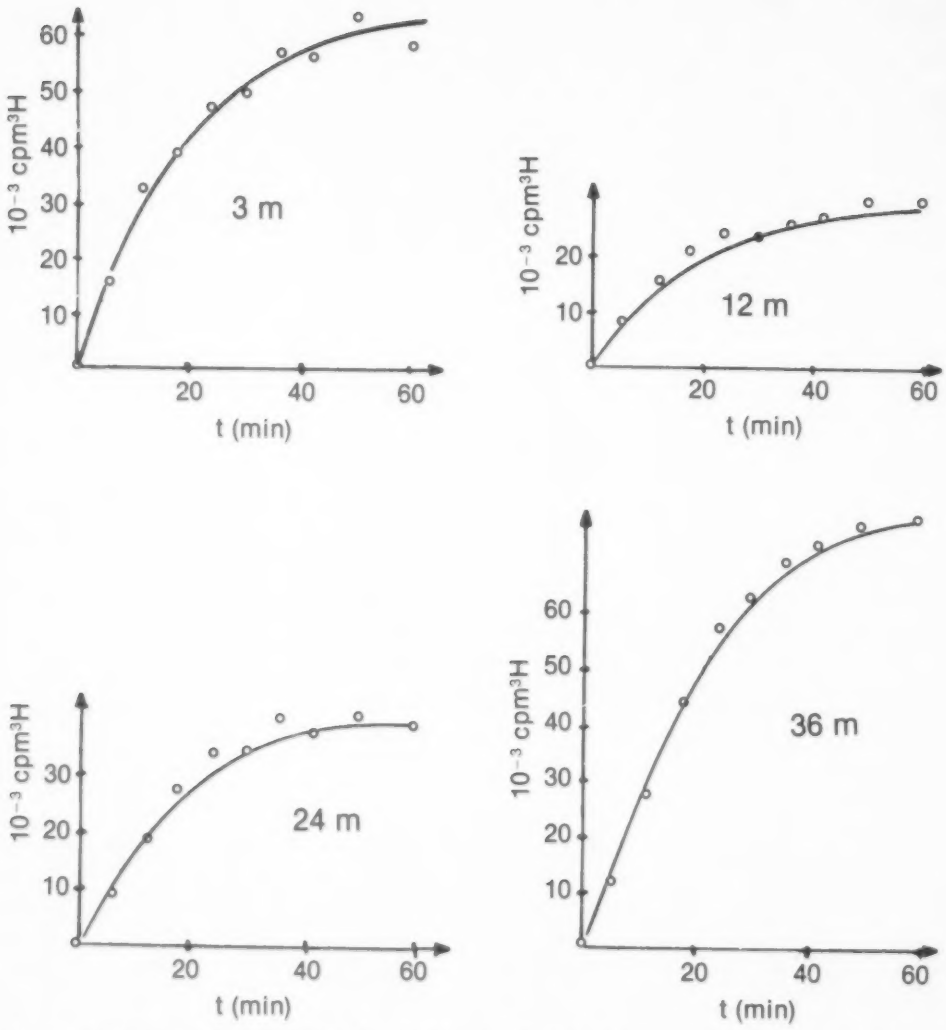

FIG. 3. *In vitro* translational activity of ribosomal fractions from 3, 12, 24, and 36 months-old Wag/Rij rats. Rat liver ribosomal fractions were incubated in a nuclease-treated reticulocyte lysate as described in Materials and Methods. The incorporation of ^{3}H-leucine into hot TCA insoluble material is plotted as a function of the incubation time. The results of a representative experiment are presented.

reasons for the increase in the protein synthesis capacity of senescent animals have been discussed elsewhere (van Bezooijen, 1977b). Concerning the mechanism involved, our results are consistent with the assumption of an increased recruitment of vacant ribosomes occuring between 12 m and 36 m. In fact, we show that a large number of single ribosomes are present at 12 m and that they disappear progressively as a function of age (Fig. 1). We also show that the translational capacity of the total ribosomal fraction gradually increases between 12 m and 36 m. This difference could be attributed to the presence at 12 m of a reserve of mRNA which could be progressively recruited in older age. As to the nature

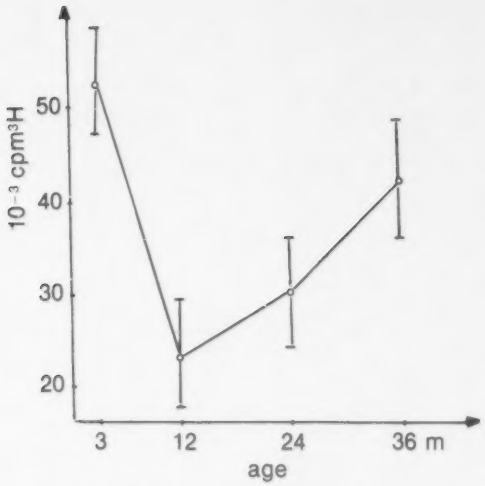

FIG. 4. Translational activity of Wag/Rij liver ribosomal fraction as a function of age. Ribosomal fraction from rats of different age groups were incubated as described in Materials and Methods. ^{3}H-leucine incorporated into hot TCA insoluble material after 60 minutes of incubation time was plotted as a function of the age. These values were respectively 53.5 ± 6.0 (4), 23.3 ± 3.0 (3), 30.5 ± 7.0 (4) and 43.0 ± 8.0 (3) (mean ± S.D., the number of experiments between parentheses).

of this mRNA, it has been shown in several systems (Ilan and Ilan, 1978) that the untranslated form of mRNA (mRNP) functions as a reserve which can be rapidly recruited upon a physiological stimulus. Zähringer *et al.* (1979), for example, demonstrated that the induction of ferritin synthesis in rat liver by injection of Fe^{++} is mediated by an increased transfer of mRNA from the inactive mRNP form towards the actively translated form. We therefore hypothesize that senescent animals use their total reserve potential for protein synthesis via a mechanism of this type. The idea of an extensive use of a protein synthesis reserve potential has been suggested by Chen *et al.* (1973); these authors showed that an increase in liver albumin synthesis can be induced in adult animals but not in senescent ones.

Acknowledgements—We thank Mrs. R.M. Wilmet-Genicot and Miss M. Jadin for excellent technical assistance. This research was carried out within the framework of the Concerted Action Programme on Cellular Ageing and Decreased Functional Capacities of Organs (EURAGE) of the European Economic Community.

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Experimental Gerontology, Vol. 17, pp. 287–293, 1982
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0531-5565/82/040287-07$03.00/0
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GANGLIOSIDE CHANGES DURING CELL AGING IN HUMAN DIPLOID FIBROBLAST TIG-1

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(*Received* 18 *November* 1981)

INTRODUCTION

HUMAN DIPLOID fibroblasts have a finite life span *in vitro* (Hayflick, 1965). This character of the human diploid fibroblasts has so far been used by many investigators as a reliable guide for the search for the mechanism of cellular aging. The significance of cell surfaces and plasma membranes in a variety of cell functions was well established. With regard to cellular aging, characteristic changes in glycoprotein components (Courtois *et al.*, 1976), cell surface charge (Bosmann *et al.*, 1976; Yamamoto *et al.*, 1980; Milo and Hart, 1976; Aizawa *et al.*, 1980), surface membrane-bound enzyme (Spataro *et al.*, 1979) and lectin binding capacity (Yamamoto *et al.*, 1977) of the human diploid fibroblasts have been studied.

Gangliosides are essential constituents of the surface membranes of mammalian cells and are known to contribute to a considerable part of the cell surface negative charge (Critchley and Vicker, 1977; Fishman and Brady, 1975). Evidence for the involvement of gangliosides in cell-surface related cellular activities has been documented (Critchley and Vicker, 1977; Fishman and Brady, 1976; Yamakawa and Nagai, 1978). However, studies on gangliosides in connection with cellular aging is meager. The situation promped us to examine whether and how this particular acidic glycolipid participates in the mechanism of aging.

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Note on abbreviations: Svennerholm symbols for gangliosides were used (Svennerholm, L. [1963] *J. Neurochem.*, **10** 613–623):

G_{M3}, NeuAcα2→3Galβ1→4Glc→Cer; G_{M2}, GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; G_{M1}, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; G_{D3}, NeuAcα2→8NeuAcα2→3Galβ1→4Glc→Cer; G_{D1a}, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc→Cer; G_{D1b}, Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glc→Cer.

In this paper we show that gangliosides of human diploid fibroblasts, TIG-1 cells, characteristically change during cell aging.

MATERIALS AND METHODS

Materials

Sephadex G-50 fine and DEAE-Sephadex A-25 were purchased from Pharmacia, Sweden. Eagle Basal Medium (Cat. No. G-13) and fetal bovine serum were from Gibco, USA. Trypsin (1:250) was obtained from Difco Lab., USA, and neuraminidase of *Cl. perfringens* (Type V) was from Sigma, USA. A glass culture dish, TD-40, was from Ikemoto Rika Company, Japan. Precoated high performance thin-layer plate of Silica gel 60 (HPTLC) was purchased from E. Merck, West Germany. All organic solvents were distilled before use.

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Cell culture

TIG-1 cell strain which is a human diploid fibroblast derived from fetal lung was established by Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan (Ohashi *et al.*, 1980). The cells were cultured in the medium of Eagle Basal Essential Medium (Gibco, Cat. No. G-13) supplemented with 10% fetal calf serum (Gibco), at 37°C under humidified 5% CO_2-95% air. When the cells reached confluency, the cells were treated by 0.25 % trypsin and the dispersed cells were subcultivated in the split ratio of 1:4. The subcultivation was performed weekly and the culture medium was changed every 3–4 days. Under these conditions TIG-1 cells had their mean life span of 54 population doubling levels (PDL).

Extraction of lipids from cells

Cells were separated from the medium and washed once by phosphate-buffered saline (PBS), pH 7.4. Washed cells were harvested mechanically by a rubber policeman into centrifuge tubes and then packed by low centrifugation. The packed cells were kept at −75°C until use. The packed cells ($1–5 \times 10^7$) were added with 3 ml of chloroform-methanol (1:1, v/v) and followed by sonication for 5 min in a bath type sonicater (200W) of Ultrasonics (USA), and then heated at 60°C for 30 min. Chloroform-methanol extract was separated by centrifugation and the pellet was reextracted with 3 ml of chloroform-methanol (1:1, v/v). This chloroform-methanol extraction was repeated three times and the extracts combined were evaporated to dryness under a stream of nitrogen. The residue was then added to 3 ml of chloroform-methanol (2:1, v/v) and sonicated for 5 min in a bath type sonicater. The clear extract separated by centrifugation was again evaporated under a stream of nitrogen and used as crude lipids.

Preparation of gangliosides

The crude lipids were dissolved in a small volume of chloroform-methanol (1:1, v/v) and the solution was applied to DEAE-Sephadex A-25 column (acetate form, 1 ml of bed volume). Non-acidic lipids were eluted with 7 ml of chloroform-methanol (1:1, v/v) solvent mixture and then acidic lipids were eluted with 7 ml of 0.2 M sodium acetate in methanol. The acidic lipid fraction was submitted to alkaline hydrolysis with 0.5 ml of 0.1 N NaOH at 37°C for 2 hr. The hydrolysate was neutralized by the addition of 0.1 ml of 0.5 N HCl and then applied to Sephadex G-50 fine column (bed volume, 20 ml) according to the method of Ando *et al.* (1978). The first 5.5 ml of water eluate was discarded and the next 8.5 ml was collected, lyophilized and used as total ganglioside fraction. The lyophilized gangliosides were dissolved in a small volume of chloroform-methanol (1:1, v/v) and an aliquot was subjected either to the determination of total sialic acid by the method of Mittinen and Takki-Luukainen (1959) or to thin-layer chromatography-densitometry to quantitate each ganglioside species. Protein content of the chloroform-methanol insoluble residue was determined by the method of Lowry *et al.* (1951).

Thin-layer chromatography and densitometry

Ganglioside solution containing about 2 μg of sialic acid was applied on a HPTLC plate and developed with chloroform-methanol-0.025% $CaCl_2$ in water (60:40:10, v/v/v). After they were developed, the plates were air-dried, sprayed with resorcinol reagent and heated on the hot-plate at 95°C for 30 min. The spots were scanned for quantitation by a Shimadzu two-wave-length scanning densitometer (model CS-910) at a wave length of 580 nm.

Neuraminidase treatment of gangliosides

A portion of total ganglioside fraction containing about 5 μg of sialic acid was dissolved in 0.1 ml of distilled water. About 0.1 unit (200 μg) of neuraminidase was added to the solution and incubated at 37°C for 2 hr. After

incubation, the reaction mixtures were added with 1 ml of chloroform-methanol (2:1, v/v) to stop the enzyme action.

Analysis of fatty acids

Total gangliosides were methanolyzed in 3% HCl-methanol in boiling water bath for 3 hr. The fatty acid methyl esters were extracted with hexane three times. The combined extracts were concentrated under a stream of nitrogen and an aliquot was injected into a column of 20% EGS on Chromosorb, W, AW (80–100 mesh, 3 mm × 2 m) at 195°C for analysis of fatty acids.

RESULTS

As shown in Fig. 1, lipid-bound sialic acid was about 2 μg per mg protein at early (PDL 15), middle (PDL 25) and late (PDL 51 and 52) passages, whereas between the middle and late passages (at PDL 38 and 44) it was twofold higher than the other passages.

Thin-layer chromatogram of gangliosides at various population doubling levels are shown in Fig. 2. G_{M3} and G_{D3} were major gangliosides. The assumption of the identity of these two gangliosides was corroborated by their disappearance after the treatment with neuraminidase from *Cl. perfringens* and concomitant appearance of the products migrating on thin-layer chromatograms at the same position as CDH (data not shown). On the chromatogram it was seen that ganglioside patterns changed during cell aging. At early passage, G_{M3}, G_{D3}, G_{M1} and other more complex gangliosides were detected, but at late passage G_{D3} and other more complex gangliosides were either decreased or not detected.

The molar percentage of the ganglioside compositions of TIG-1 cells during cell aging is shown in Table 1. During cell aging, the molar percentage of G_{M3} was increased from about 70% to over 90%. In contrast to the increase of G_{M3}, the other gangliosides decreased or were no more detectable, particularly in the late passage. As reported previously (Ohashi *et al.*, 1980), the saturation density of TIG-1 cells was changed during cell aging. It had to be ascertained whether or not ganglioside composition changed depending on cell density. Therefore, ganglioside composition was compared between 3-day-old and 6-day-old cultured cells at early passage (PDL 17), whose cell densities were

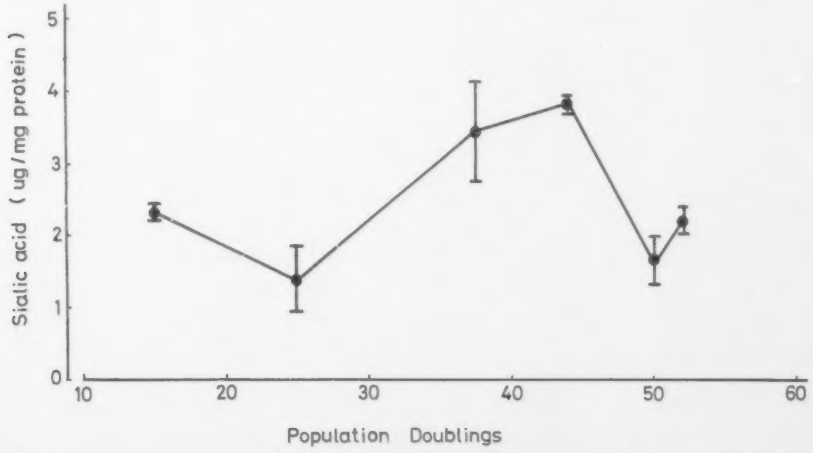

FIG. 1. Change in lipid-bound sialic aid of TIG-1 cells during cell aging. Sialic acid determination was performed in triplicate and the data was represented as mean ± S.D.

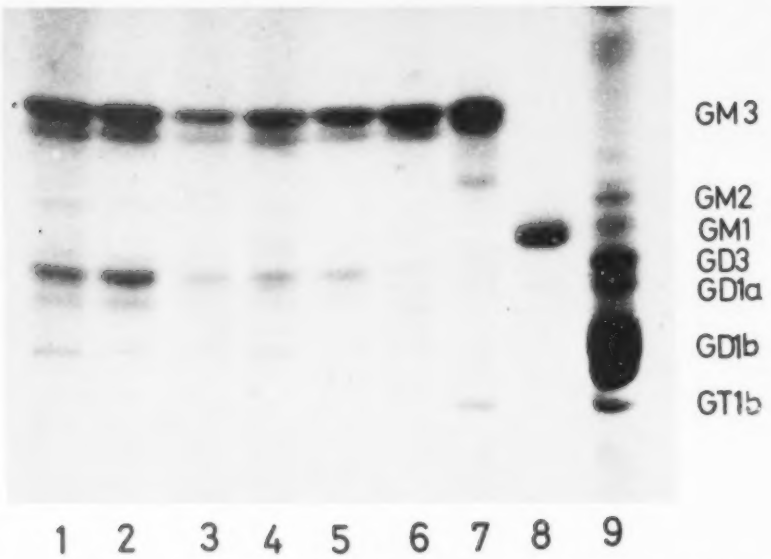

FIG. 2. Thin-layer chromatogram of gangliosides of TIG-1 cells at various PDLs. The developing solvent system was chloroform-methanol-0.025% $CaCl_2$ in water (60:40:10, v/v/v). After development, resorcinol reagent was sprayed. 1, PDL 15; 2, PDL 25; 3, PDL 38; 4, PDL 44; 5, PDL 50; 6, PDL 52; 7, reference standard, equine erythrocyte G_{M3}; 8, reference standard, bovine brain G_{M1}; 9, a ganglioside fraction from bovine brain as reference standard.

$3.6 \times 10^4/cm^2$ and $6.6 \times 10^4/cm^2$, respectively. As shown in Table 1, molar percentage compositions of gangliosides in these cells were not different from each other. A similar result was obtained when the ganglioside compositions at the late passages, PDL 44 (6-day-old culture) and PDL 46 (2-week-old culture), whose cell densities were $3.0 \times 10^4/cm^2$ and $4.3 \times 10^4/cm^2$, respectively, were compared.

Fatty acid compositions of total gangliosides were not different between younger and older cells, except that oleic acid tended to be higher in younger cells and arachidonic acid lower in younger cells (Table 2).

DISCUSSION

The ganglioside compositions of cultured human diploid fibroblasts have been analyzed by many investigators (Hakomori, 1970; Lingwood and Hakomori, 1977; Dawson *et al.*, 1972; Fishman *et al.*, 1978; Bach *et al.*, 1975). All of these reports showed that the sialoglycolipids of cultured human fibroblasts were predominantly the gangliosides of G_{M3}, G_{D3} and G_{M1}, although the relative ganglioside compositions were found to be different in these various investigations. In this study it was also found that TIG-1 cells of human diploid fibroblasts derived from a fetal lung had these major gangliosides and additional minor gangliosides. However, the ganglioside compositions and their cellular amounts of TIG-1 cells showed interesting changes during cell aging. As reported previously (Ohashi *et al.*, 1980), the saturation density of TIG-1 cells was changed during cell aging. Since it is known the glycolipid concentration and pattern may depend on the cell density, we also

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TABLE 1. GANGLIOSIDE COMPOSITION OF TIG-1 CELLS DURING CELL AGING AT DIFFERENT CELL DENSITY.

PDL[a]	Cell density[c]	molar percent				
		GM_3	GM_2	GD_3	GD_{1a}	GD_{1b}
15 (6D)[b]	$1.0 \times 10^5/cm^2$	69.9 ± 3.8	3.2 ± 1.5	20.6 ± 2.7	3.8 ± 1.1	2.5 ± 0.7
17 (3D)	3.6×10^4	71.5 ± 0.7	9.9 ± 0.1	17.3 ± 0.2	–	–
17 (6D)	6.6×10^4	72.1 ± 5.1	10.0 ± 1.3	20.0 ± 2.5	1.4 ± 0.2	–
25 (6D)	7.2×10^4	71.3 ± 2.3	3.4 ± 1.9	17.7 ± 1.1	6.2 ± 0.8	–
38 (6D)	3.8×10^4	79.3 ± 2.1	5.8 ± 3.1	13.1 ± 1.1	3.0 ± 1.2	–
44 (6D)	3.0×10^4	81.5 ± 0.7	–	18.5 ± 0.7	–	–
46 (2W)	4.3×10^4	82.1 ± 5.2	–	15.6 ± 3.7	–	–
47 (6D)	3.0×10^4	84.8	–	14.2	–	–
50 (6D)	3.4×10^4	87.5 ± 1.3	n.d.	12.4 ± 1.5	–	
52 (11D)	3.0×10^4	91.6 ± 1.5	n.d.	3.3 ± 0.4	4.9 ± 1.3	–

[a]PDL = population doubling levels; [b]Culture days (D) and weeks (W); [c]Cell density was counted under the Olympas light microscope model EHS after tripsinization. All determinations were triplicate and shown as mean ± S.D., except that the determination of PDL 47 (6D) was from a single experiment. – = not determined due to trace amounts; n.d. = not detected.

analyzed the gangliosides of TIG-1 cells of either early passage or late passage at high and low cell densities. The results showed that in the ganglioside compositions of TIG-1 cells at either early or late passages, there was no apparent difference between high cell density and low cell density. These results suggest that the increase in ganglioside G_{M3} and the decrease of the other gangliosides in their compositions during cell aging were dependent on cell senescence. This also suggests that different values reported by several laboratories on the ganglioside compositions of cultured human dipoid fibroblasts could be related to the difference of the cell passage level which they used.

Hakomori, using human diploid fibroblast 8166 cell line, reported that at high cell density certain gangliosides accumulated and that G_{D3} increased at confluency as compared to the growing stage (Hakomori, 1970). In the present study, however, we could not observe an obvious difference in ganglioside compositions depending on cell density either at early passage or late passage. A more precise examination of the dependency of ganglioside pattern of TIG-1 cells on cell density is now in progress.

It was also found in this study that not only the pattern but also the concentrations of the ganglioside changed during cell aging. At the early (PDL 15), middle (PDL 25) and late passages (PDL 51 and 52) there was about 2 μg of lipid-bound sialic acid per mg protein, whereas it was about twofold higher between the middle and late passages (at PDL

TABLE 2. FATTY ACID COMPOSITION OF TOTAL GANGLIOSIDES FROM TIG-1 CELLS.

	PDL 15	PDL 52
16:0	16.1 ± 1.4	16.7 ± 2.5
18:0	39.6 ± 2.3	36.6 ± 0.9
18:1	28.7 ± 7.0	19.8 ± 1.2
18:2	3.5 ± 0.4	4.5 ± 0.5
20:4	6.6 ± 4.7	12.5 ± 1.7

The values were shown as mean ± S.D.

38 and 44). The observed increase of ganglioside concentration per mg protein may be significantly related to the change of the cell's potential to divide and cell senescence, because PDL 38 and 44 correspond with early phase III according to Hayflick's nomination (Hayflick, 1965), and after that, cell doubling time becomes slower and the increase of cellular protein content accelerates (Ohashi *et al.*, 1980). Except for this increase, the concentration of the ganglioside was found to be similar to the values which were reported by Bach *et al.* (1975) for their cultured human skin fibroblasts, but higher than the 1.09 n mol of G_{M3} per mg protein that was found by Dawson in human skin fibroblasts (Dawson *et al.*, 1972) as well as the 3.1 n mol of this ganlgioside per mg protein that was found by Fishman *et al.* (1977) in human diploid fibroblasts from normal male. In both reports ganlgioside G_{M3} constituted about 90% of the total sialoglycolipid.

As described previously (Ohashi *et al.*, 1980), TIG-1 cells change in volume during cell aging with about a twofold increase in the concentration of protein. Therefore, if the lipid-bound sialic acid was expressed per cell, the ganglioside values at late passages might be at almost the same level of that at PDL 38 and 44. This suggests that the lipid-bound sialic acid per cell was increased after the middle passage in correlation with the increase of cell volume.

Bosmann *et al.* (1976) reported that the cell electrophoretic mobility of WI-38 cells decreased during cell aging and, for this reason, suggested a decrease in the negative charge of cell surface. Recently, Yamamoto *et al.* (1980) also reported that TIG-1 cells showed a decrease in electrophoretic mobilities during cell aging. Milo and Hart (1976) reported that the total sialic acid content of the plasma membrane of WI-38 cells per mg protein which was ascribed to both glycoprotein and glycolipid showed a decrease during cell aging. On the other hand, Yamamoto *et al.* (1980) found a decrease of hyaluronic acid on the cell surface of TIG-1 cells during cell aging. In addition, Aizawa *et al.* (1980) observed a decrease of the cell surface negative charge in parallel with cell aging from the early phases of cell passage of TIG-1 cells using a concanavalin A-mediated red blood cell adsorption method. At present it is not clear whether or to what extent the decrease in electrophoretic mobilities of WI-38 and TIG-1 cells during cell aging can be related to a change in lipid-bound sialic acid.

It is to be noted that gangliosides with more complex structures than G_{M3} remarkably decreased or were no more detected during cell aging of fetal-lung-derived human diploid fibroblasts, TIG-1 cells, suggesting an interesting alteration of the metabolic background of gangliosides.

SUMMARY

Gangliosides of human diploid fibroblast, TIG-1, derived from fetal lung were analyzed during cell aging. The major gangliosides of TIG-1 cells were G_{M3} and G_{D3}. During cell aging, G_{M3} and other gangliosides were decreased or below detection level. Lipid-bound sialic acid per mg protein was 2 μg at the early, middle and late passages, but between the middle and late passage it was twofold higher than the other passages.

Acknowledgement—This work was supported in part by a grant from the Ministry of Education, Japan.

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Experimental Gerontology, Vol. 17, pp. 295–303, 1982
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PHYSIOLOGICAL AND BEHAVIORAL CORRELATES OF LIFESPAN IN AGED C57BL/6J MICE

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(*Received* 2 *June* 1981)

GERONTOLOGISTS HAVE long expressed interest in the intracohort variance observed in the results of tests administered across the adult lifespan (Benjamin, 1947; Birren, 1959; Comfort, 1969; Heron and Chown, 1967; McFarland, 1973). Two approaches to the quantitative study of this issue can be identified. One approach views the variability in test scores among individuals of similar chronological age as evidence that aging proceeds at highly individualized rates (Burney, 1972; Damon, 1972; Dirken, 1972; Fozard and Thomas, 1975; Furukawa, *et al.*, 1975; Heikkinen, *et al.*, 1974; Webster and Logie, 1976). In these studies, chronological age is viewed merely as an index of an underlying process, which has been called biological aging, physiological aging, or functional aging. The use of multiple regression techniques has been the prevalent strategy to emerge in these studies to assess the variation in aging rates. Scores are obtained from a number of psychological and biomedical tests, and the resulting data are submitted to multiple regression to yield an estimate of the individual's biological age, which is correlated with chronological age but viewed as a more accurate reflection of potential lifespan.

Costa and McCrae (1980) have offered several major criticisms of this approach, including the inattention given to "regression towards the mean" and the lack of validation studies in other than clinical populations. Most critically, however, they note the tautology implied in the strategy. While the rationale of these studies has been to construct indices of aging that are more meaningful than chronological age, the validity of the regression is

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usually judged by how well chronological age can be predicted. The major shortcoming of this strategy, then, has been its failure to identify an appropriate criterion for regression. If one individual is judged biologically younger than another, it may be reasonable to deduce that the former is probably further from death than the latter. Therefore, the construction of tests of biological age should not be based entirely on their ability to predict chronological age. Their ability to predict lifespan should be considered as well.

The latter strategy constitutes the second approach to the study of intracohort variance, namely, the prediction of lifespan through multivariate techniques. The focus of many such studies is on survival, since the primary interest is in the identification of morbidity and its consequences in clinical populations (Baer and Gaitz, 1971; Garrity and Klein, 1971; Lieberman, 1971; Linn, *et al.*, 1971). Yet there have been studies of relatively healthy samples that have produced regression analyses based on psychological, social, and biomedical variables, and these, too, have proven capable of accounting for significant amounts of the variance in the longevity of their subjects (Bartko, *et al.*, 1971; Botwinick, *et al.*, 1978; Palmore, 1971; Pfeiffer, 1971; Riegel 1971).

All the above studies have involved human subjects. There has been sparse application of multivariate techniques in the search for correlates of lifespan in laboratory animals. Using nutritional and growth data from laboratory rats, Ross, *et al.* (1976) have conducted lifespan studies demonstrating the power of this analytic procedure. The potential for explaining significant amounts of the variance in lifespan through test results remains, and it should be enhanced in studies of nonhumans because of the greater potential for controlling extraneous sources of variance, such as accidents, health factors, and attrition. Moreover, there are many practical implications in determining predictors of lifespan in laboratory animals. Of particular interest is the identification of systems most vulnerable to aging. Furthermore, with accurate predictors of lifespan the success of experiments that manipulate longevity could be assessed much earlier in the lifespan.

With these objectives in mind, the present study sought to identify correlates of lifespan in the male C57BL/6J mouse. The strategy was to determine if this could be accomplished in a cohort of aged animals, the rationale being that death would be imminent and that variance associated with physiological and behavioral functioning would be greater than in younger cohorts. A battery of tests assessing both physiological and behavioral traits was selected on the basis that each component had previously shown a significant relationship, either linear or quadratic, with chronological age in this strain of mouse. The test measurements were analyzed to determine whether significant relationships existed with lifespan.

MATERIALS AND METHODS

We began testing a cohort of 29 male, experimentally naive, C57BL/6J mice at 905 ± 5 days of age, which was beyond the estimated mean survival time of 827 days reported by Goodrick (1975). These animals were approximately 8 weeks old when obtained from the Division of Animal Resources of the Jackson Laboratory. They had been housed five to a cage in plastic cages with wire tops and with wood shavings for bedding. At the time of testing, the number per cage ranged from one to four. During testing, the cages were located in a vivarium maintained on a 12-h light: 12-h dark cycle at a temperature of 22 ± 2°C. A radio provided background noise for 18 hours daily. The animals were tested in rooms adjacent to the vivarium. Approximately 2 weeks after completion of testing, the mice were returned to the original colony where they remained until their death. The conditions in the colony were identical to the vivarium except for the background noise. The mice were maintained on Old-Guilford 96W Chow (vitamin K fortified) and acidified water (pH 3). Fresh cages, food, and water were provided weekly. All testing was conducted during the light portion of the light-dark cycle.

Over the course of 7 days, the following measurements were obtained sequentially:

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Body weight (BW)

Each animal was weighed (to the nearest 0.1 gram) on Day 1.

Activity (ACT)

Exploratory activity in this strain is known to decline with age (Sprott and Eleftheriou, 1974). For this study, the exploratory activity of each animal in an open field was measured on Day 1 during one 15-min trial as recorded by movement on a jiggle platform (BRS/LVE). Each mouse was removed from its home cage and placed into a plastic cage (29 × 17 × 13 cm) resting on the stage of the jiggle platform. The floor of the plastic cage was lined with a white paper towel, which was changed at the end of each trial. The entire apparatus was located in a box (39 × 37 × 48.5 cm) that blocked the view of extraneous activity in the test room. Illumination was supplied by overhead fluorescent lighting. Between each trial, the jiggle platform was calibrated with the use of a metronome set at 225 beats per min. The variable of interest was the total number of activity counts recorded.

Rotorod performance (ROT)

On Day 2, the motor coordination of each mouse was tested in terms of its ability to remain on a Plexiglas rod (11.5 cm in diameter) rotating at 1 rpm. This ability has also been observed to decline with age (Sprott, 1975). For the test, each animal was removed from its home cage and placed into a metal pan with a white paper towel for flooring. About 60 sec afterwards, the mouse was placed by the tail onto the rotating rod and allowed to gain footing. If the animal lost its footing, a fall of 27.5 cm into the metal pan resulted. If the mouse remained on the rod for 90 sec, it was gently removed and placed into the metal tray. Thirty trials were administered with an intertrial interval of approximately 60 sec. Between each trial the rod was wiped dry of urine. The variable of interest was mean time on the rod per trial.

Passive avoidance learning (PAL)

On Day 5, the ability of each animal to learn a simple inhibitory avoidance task was tested by using a similar apparatus and procedure described previously (Sprott, 1978). Basically, the apparatus consisted of a Plexiglas chamber with a stainless steel grid floor, wired for electric shock, and a shelf onto which the mouse was placed for each trial. When the mouse stepped down from the shelf with all four paws onto the grid, the shelf was retracted against the wall, and the grid was electrified with 0.1 mA shock for 3 sec. The animal was removed quickly with forceps from the chamber and placed into a holding cage for a 60–90 sec intertrial interval. If, on the other hand, the mouse remained on the shelf for 60 sec, it was simply removed from the chamber to the holding cage for a 60–90 sec intertrial interval. Each animal was tested for acquisition of this response as measured by three consecutive trials during which it remained on the shelf for the entire 60 sec, within a total of 25 trials. After each trial the grids were wiped dry of urine, and a fresh paper towel was placed underneath the shock chamber. The variable of interest was the number of trials to criterion.

Tightwire performance (TW)

On Day 7, each animal's strength and agility were tested by its ability to remain suspended on a tightwire (1.0 mm in diameter) strung between two ring stands as described previously (Miquel and Blasco, 1978). The animal's weight was moved below the wire before the mouse was freed. If the mouse lost its grip, a fall of 30 cm into a cotton-filled pad resulted. Five trials were given with an intertrial interval of 10 sec. The variable of interest was mean time on the tightwire over three trials.

Hematocrit (HC) and Hemoglobin (HG)

Immediately after the TW test, a sample of blood was drawn from the retroorbital sinus using a standard 70 μl heparinized capillary tube. After 5.0 μl of blood were removed for determination of hemoglobin concentration, the tube was centrifuged in an IEC micro-hematocrit centrifuge for 5 min. The hematocrit (HC), or percentage of blood volume that was packed erythrocytes, was measured. The 5.0 μl blood sample was mixed thoroughly with 2.0 μl Drabkin's reagent (Fisher Scientific D-120), and hemoglobin concentration (HG) was read on a Bausch and Lomb Spectronic 70 spectrophotometer. The concentration was calculated in grams hemoglobin per dl, and this was the variable of interest. These variables were also known to decline with age in mice (Leuenberger and Kunstýř, 1976).

Collagen denaturation rate (COL)

The denaturation rate of collagen in the tail tendon was measured on Day 7, according to a procedure described previously (Harrison and Archer, 1978). Portions of a tail tendon were removed from each animal. Three in-

dividual fibers were removed from the tendon; each was tied between sutures that had a 2g weight attached to one end; and these preparations were suspended in a urea bath (7*M*, pH 7.5, 45.0 ± 0.1°C). The variable of interest was the mean denaturation time of three fibers from the tendon.

Lifespan (LS)

After completion of testing, the survival of the mice was checked three times a week. Dead animals were removed upon discovery. Because the exact date of death was difficult to determine, lifespan was calculated to the nearest week.

RESULTS

The range of survival beyond testing was 1–22 weeks, and the mean ± SEM lifespan of the cohort was 139.3 ± 1.2 weeks. For comparative purposes, the independent variables were grouped into two sets: the behavioral set, i.e., variables ACT, ROT, PAL, and TW, and the physiological set, i.e., variables BW, COL, HC, and HG. The intercorrelations of the eight independent variables are presented in Table 1. With the exception of the significant correlation between HC and HG, the variables were free from multicolinearity—both within and between each variable set (Gordon, 1968). Since the close association between variables HC and HG was expected, given the relatedness of the measures, and since no direct comparison between the two variables was intended, neither HC nor HG was eliminated from subsequent multivariate analyses.

To establish whether the regression of each independent variable on lifespan was linear, separate trend analyses for each of the behavioral and physiological sets were performed. The results are summarized in Table 2.

For three of the physiological variables—HC, HG, and COL—significant linear relationships with lifespan emerged. BW showed no relationship with lifespan, either linear or curvilinear. Mice with the highest HC counts tended to live the longest, while those with the lowest HC counts tended to be the shortest lived. The relationship between HG and lifespan paralleled that of HC and lifespan. Less expected, however, was the positive linear relationship between COL and lifespan. Mice with the longest collagen denaturation times, indicating older collagen, tended to live the longest, while the opposite was true for mice with the shortest collagen denaturation times.

Of the four behavioral variables, only two, PAL and ACT, showed significant relationships with lifespan, each representing quadratic functions. The quadratic relationship between ACT and lifespan is depicted in Figure 1. The relationship between lifespan and

TABLE 1. INTERCORRELATION MATRIX OF PHYSIOLOGICAL AND BEHAVIORAL VARIABLES

	Pearson Correlation Coefficients							
Variable	BW	HC	HG	COL	ACT	ROT	TW	PAL
BW	1.00							
HC	.20	1.00						
HG	.16	.88*	1.00					
COL	.09	.26	.16	1.00				
ACT	.27	.26	.25	−.14	1.00			
ROT	−.03	.18	.18	−.18	.07	1.00		
TW	−.06	−.01	.08	−.18	.01	.09	1.00	
PAL	.34	−.10	−.16	.26	−.26	−.18	.05	1.00

*$p < .01$.

PAL was similar. Both trends were inverted-U shaped functions, i.e., low scores and high scores were associated with the shortest lived mice, while moderate scores were recorded by longest lived mice.

Having established the nature of the relationships between these independent variables and lifespan, it was then possible to examine their collective contribution to the variance accounted for in the criterion, lifespan, by use of multiple regression techniques. Presented in Table 3 are the results of a stepwise multiple linear regression analysis in which HC, HG, and COL were added to the regression equation until no significant increment in the multiple correlation, R, was obtained (Kerlinger and Pedhazur, 1973). Because HC had the highest zero-order correlation with lifespan, it entered first into the regression equation. COL entered into the equation next, adding a significant increment in the

TABLE 2. SUMMARY OF THE LINEAR AND CURVILINEAR REGRESSION OF LIFESPAN ON EACH INDEPENDENT VARIABLE

Variable	Source[a]	R[b]	R^2[c]	df	F[d]
		Physiological variables			
BW					
	Linear	.16	.03	1/27	<1
	Quadratic	.33	.11	1/26	2.32
HC					
	Linear	.49	.24	1/27	8.10**
	Quadratic	.49	.24	1/26	<1
HG					
	Linear	.47	.22	1/27	7.70**
	Quadratic	.50	.25	1/26	<1
COL					
	Linear	.46	.21	1/27	7.00*
	Quadratic	.48	.23	1/26	<1
		Behavioral variables			
PAL					
	Linear	.30	.09	1/27	3.96
	Quadratic	.63	.40	1/26	13.61***
TW					
	Linear	.17	.03	1/27	<1
	Quadratic	.32	.10	1/26	2.07
ROT					
	Linear	.30	.09	1/27	2.57
	Quadratic	.31	.09	1/26	<1
ACT					
	Linear	.08	.01	1/27	<1
	Quadratic	.52	.27	1/26	12.20***

[a]The addition of the cubic and quintic components in each regression equation yielded nonsignificant increments in R^2.

[b]R is the coefficient of multiple correlation.

[c]R^2 expresses the proportion of variance accounted for in the criterion, lifespan.

[d]F ratios reported are for the increment. F ratios for the linear components are based on the error terms associated with the quadratic polynomials.

*$p \leq .05$
**$p \leq .01$
***$p \leq .001$

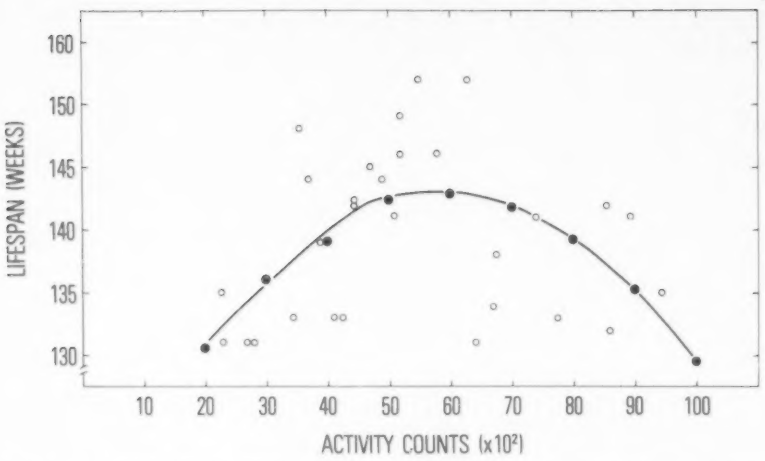

FIG. 1. The quadratic relationship between lifespan and exploratory activity in aged C57BL/6J mice.

variance accounted for in lifespan. The inclusion of HG into the regression equation yielded a nonsignificant increment in the multiple correlation, $F < 1$, which, given the high correlation between HC and HG, would be expected on the basis of the multiple regression model. Collectively, then, HC and COL accounted for more than a third of the variance in lifespan.

Finally, a multiple regression analysis was performed in which lifespan was regressed onto the second-order poly-nomials of the behavioral measures PAL and ACT simultaneously. The resulting multiple correlation was 0.66, $F(4,24 = 4.66, p < .007$. Thus, the curvilinear regression of these variables on lifespan accounted for over 40% of the variance in lifespan, $R^2 = 0.44$.

DISCUSSION

Significant relationships with lifespan were found for both physiological and behavioral variables in this cohort of aged mice. Using multiple regression techniques, it was possible

TABLE 3. SUMMARY OF THE STEPWISE MULTIPLE REGRESSION OF LIFESPAN ON THE PHYSIOLOGICAL PREDICTORS

Step	Variable entered	R	R^2	df	F to Enter[a]	F[b]
1	HC	.49	.24	1/27	8.38**	
				1/27		8.38**
2	COL	.59	.35	1/26	4.68*	
				2/26		7.10**
3	HG	.61	.38	1/25	<1	
				3/25		5.00**

[a]The "F to Enter" is a test of the significance of the increment in the proportion of variance accounted for by a given variable when entered into the regression equation.

[b]The F ratio for the overall R at each step.

*$p \leq .05$

**$p \leq .01$

to assess the collective contribution of the variables of each set to the observed variance in lifespan. Each of the resulting regression analyses clearly indicated that a substantial proportion of the observed variation in lifespan was explainable.

The trend and direction of some of the relationships require additional discussion. Positive relationships between lifespan and the physiological variables, HC and HG, had been predicted on the basis of the positive relationships with chronological age observed previously (Leuenberger and Kunstýř, 1976). This prediction was confirmed, i.e., the higher the blood parameters, the longer the survival of the animal. The relationship between COL and lifespan was also significant but in the direction opposite that observed in its relationship to chronological age in this mouse strain (Harrison and Archer, 1978; Harrison *et al.*, 1978). The results indicated that the longest lived animals tended to be the ones with the "oldest" collagen. This paradox can possibly be explained in the findings of Everitt (1969). The collagen fibers of 885 ± 11 day old rats surviving less than 100 days denatured 30 to 40% faster than fibers of rats that lived longer than 100 days. Our mice were old enough that those nearer to death may have had faster denaturing, or "younger" appearing, collagen.

The change in direction of a lifespan correlate is not unprecedented. For example, a previous study observed that body weight in humans was positively related to lifespan at older ages, although an inverse relationship had been expected on the basis of established research (Bartko *et al.*, 1971). In the present study, BW was the only physiological variable that failed to show a significant relationship with lifespan. This virtual absence of association was surprising since Sacher and Duffy (1979) had observed a significant quadratic relationship between BW and lifespan in aged mice of several strains. In contrast to the previous study, however, our analysis was based on data from individual animals of advanced age within one strain, rather than on strain means.

While no linear relationships emerged between lifespan and the behavioral variables, two quadratic relationships were observed. The significant quadratic relationships obtained between lifespan and PAL and ACT were interpretable. The longest lived animals required a moderate number of trials to reach criterion in the PAL task and exhibited a moderate level of activity in the open field task. It seemed that very low scores in the PAL task were made by mice tending to have long initial latencies in stepping down from the shelf. This might be an index of very low activity, or possibly feebleness. Conversely, very high scores on the PAL task were made by animals that were hyperactive and that frequently fell from the shelf. Animals exhibiting a moderate number of trials to criterion were those exhibiting short latencies in the initial step-down from the shelf; however, these mice learned to remain on the shelf after a few trials. In regard to the nonlinear relationship of ACT with lifespan, it was expected that hypoactive animals would tend toward shorter survivals; however, the short survival time of the most active mice made sense only from anecdotal observations. Without documentation, we had observed in our colony that, prior to death, hyperactivity occurred in many mice, particularly those that suffered severe hair loss. This observation was not confirmed, however, in a study of survival in mice after a prolonged period of runwheel activity. Wax and Goodrick (1978) reported that a linear relationship was obtained between activity and survival i.e., mice with the shortest survival times had the lowest levels of wheel-running activity. Thus, while the findings of our study are suggestive, they require further documentation.

That correlates of lifespan were found in mice of essentially identical genotypes underscores the utility of this analytical approach in longitudinal studies using animal models.

This demonstration of an aging phenotype supports previous evidence of the impact of environmental factors upon genetically programmed aging in these animals (Goodrick, 1978). Furthermore, the general lack of interrelatedness among the variables reinforces the argument that aging must be viewed as a multidimensional phenomenon (Comfort, 1969). Therefore, the greatest potential for accurate prediction of the rate of aging will be found in a multivariate approach.

Whether the explainable variance in lifespan can be interpreted as a measure of survival from morbidity occurring at this advanced age is a central remaining question. In future studies, particular emphasis should be given to documenting pathological changes and to demonstrating significant relationships at much younger ages than presently observed when morbidity is less probable. The objective would be a regression analysis using both physiological and behavioral variables to predict survival with a high degree of accuracy over a substantial portion of the lifespan. To this end, much larger sample sizes and a greater range of tests will have to be employed. In addition, testing of the individuals on multiple occasions is recommended to aid in determining the evolution of apparent direction shifts in the relationships and also of nonlinear trends in the parameters studied. To this end, it should be recognized that lifespan is not the ultimate criterion for the assessment of biological aging. It is possible that an individual or a genotype might be especially vulnerable to a particular lethal pathogen that might result in death before most systems age significantly. Perhaps, then, the ultimate criterion should be one that offers in addition to the prediction of lifespan the predictability of the rate of aging in several systems. The present study has demonstrated the potential for predicting, with a few simple tests, lifespan in mice.

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SUMMARY

Physiological and behavioral measurements were made in a cohort of 29-month-old male C57BL/6J mice to determine whether any correlated significantly with lifespan. Significant linear relationships with lifespan were found among the physiological measures, including hematocrit and hemoglobin levels and collagen denaturation rate; however, body weight failed to be a significant predictor of survival. Among the behavioral variables studied, significant quadratic relationships with lifespan were found for exploratory activity and passive avoidance learning, while performance on a rotorod and a tightwire showed no significant relationships with lifespan. Through the use of multiple regression techniques, about one-third of the variance in lifespan could be explained by a combination of physiological variables, and about two-fifths could be explained by a combination of behavioral variables.

Acknowledgements—This research was supported in part by the following grants: NIH grant AG-00250, awarded to R.L. Sprott; NIH grants AG-01755 and AG-00594, awarded to D.E. Harrison; and NIH postdoctoral fellowship, AG-05171, awarded to D.K. Ingram, while he was a postdoctoral associate at the Jackson Laboratory. The Jackson Laboratory is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

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Experimental Gerontology, Vol. 17, pp. 305–317, 1982
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ON THE ERROR THEORIES OF AGING

A Review of the Experimental Data

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(*Received* 2 *October* 1981)

Abstract—Error theories are exerting a profound influence on the study of aging because they offer a coherent and simple explanation for the cause of senescent death. However its experimental testing is not a straightforward matter. The available experimental evidence is reviewed here.

INTRODUCTION

THERE ARE almost as many theories of aging as there are investigators (Sonneborn, 1978). Nevertheless, the favored theories can be grouped into two categories: (1) the programmed aging theories: aging and death are developmental events, like the resorption of gills, tails and larval muscles during amphibian morphogenesis (Lints, 1978; Saunders, 1966); and (2) the information loss theories: aging comes from a progressive accumulation of faulty macromolecules up to the point where some cells of an organism become so metabolically crippled that systemic death results.

Faulty macromolecules can accumulate through two different mechanisms: (a) "wear and tear" (think of the mechanisms through which man-made instruments eventually cease to function) and (b) synthesis errors during the polymerization of DNA, RNA or proteins from their respective building blocks.

"Wear and tear" describes any damage to a molecule due to its natural thermodynamic instability (Robinson, 1979; Strehler, 1977) or to the nature of the environment (cosmic rays, β and γ rays due to intrinsic radioactive decay, presence of superoxides and of other highly reactive free radicals as a result of respiration and normal metabolism, fast hydrolysis of glutaminyl and asparaginyl groups due to the high local concentration of water, etc.). The "error theories" of aging simply recognize the fact that DNA, RNA and protein synthesis (but not, for example, polysaccharide synthesis) are intimately connected to each other, both forward and backward. Protein synthesis requires an RNA template, RNA synthesis needs both a DNA template and a protein enzyme; finally, closing the circle, DNA replication involves protein enzymes (Watson, 1976). Obviously, errors in the template will yield to defective enzymes which may result in more errors in the templates (and *mutatis mutandis*), etc. until the number of errors reaches proportions incompatible

with life. Alternatively, translation errors committed by a normal ribosome may lead to the synthesis of a faulty ribosome (because the ribosome is itself made of proteins synthesized by another ribosome) which leads to worse ribosomes, etc. Since the error increase can be an exponential function of time (Orgel, 1963; Menninger, 1977; Edelman and Gallant, 1977), error theories are sometimes called "error catastrophe" theories (Orgel, 1973; Kirkwood, 1980).

The accuracy of DNA replication is 4 to 7 orders of magnitude higher than that of DNA translation (Drake, 1969; Dove, 1968; Loftfield and Vanderjagt, 1972; Kimura and Ohta, 1973). Correct DNA translation can be divided into three consecutive steps (Watson, 1976): (1) accurate DNA transcription into messenger (m)RNA, ribosomal (r)RNA and transfer (t)RNA; (2) linkage of every amino acid species to its tRNA mate, and (3) accurate mRNA codon - aminoacyl-tRNA anticodon recognition by the ribosome followed by accurate 3-nucleotide mRNA movement. Infidelities in steps (1), (2) and (3) have not yet been separately measured *in vivo*. However *in vitro* experiments suggest that step (3) is the worst offender and perhaps by one order of magnitude or so (Losick and Chamberlin, 1974; Mori *et al.*, 1979). Thus most proponents of error theories of aging postulate that the cause of senescent death rests on the intrinsically fallible nature of the ribosome and on the incapacity of some cells to prevent subsequent error amplification (Orgel, 1973; Kirkwood, 1980) through, for example, rapid detection and destruction of most defective proteins (Etlinger and Goldberg, 1977; Capecchi *et al.*, 1974).

The cell can reduce the risk of "wear and tear" through a fast turnover of its macromolecules (Zak *et al.*, 1979; Hirsch and Hiatt, 1966). On the other hand, fast turnover cannot reduce the rate of error production during the synthesis of DNA, RNA and protein.

EXPERIMENTAL DATA RELEVANT TO THE ERROR THEORIES OF AGING

The error theories of aging have stimulated many experiments and speculations which, together, are helping to clarify the problem (Kirkwood, 1980). A fundamental question to answer is the following. Does translation fidelity decrease during senescence? Current evidence on this issue is reviewed below. Note that very few of the experiments have been done on animals, and virtually none on human beings. On the other hand, many have involved human diploid fibroblasts grown *in vitro*. While the study of, for example, an old rat is a proper study of mammalian aging, that of diploid fibroblasts grown *in vitro* is a study not of aging, but *of a model of aging*. Such studies are useful and relevant, but they can only provide conjectures as to the causes of mammalian senescence. Thus, the following review will deal mostly with studies of fibroblasts, not because it is biased in favor of such studies and against animal or human studies, but simply because, so far, the fibroblast model system has been the focus for most experiments on the issue of the connection between translation errors and aging.

Does fidelity of translation decrease with aging?

Evidence on this point has been sought by examining the fidelity of synthesis of a variety of proteins, as judged by their physical characteristics, by the degree of incorporation of incorrect amino acids or analogues, or by the accumulation of mutant phenotypes.

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1. Errors of synthesis of viral protein. It has been stated (Lints, 1978) that one of the strongest pieces of evidence against the error theories is the work of Holland *et al.* (1973). These workers reasoned that viral infection of old fibroblast cultures could, according to error theories, increase the generation of viral mutants. The advantage of their assay is that even a 2- or 3-fold increase in mutation rate should be detectable. They observed that mutation rates (to guanidine resistance) of poliovirus grown in late- and middle-passage fibroblasts were similar. If universal, and if it also applies to old animals, this result can be damaging to both the error theories and the somatic mutation theory (a theory which I included under the label "wear and tear"). As pointed out by the authors themselves, their single observation cannot even disprove the error theories as the mechanism of *in vitro* sterilization (or "aging") of human fibroblasts. The reasons are numerous.

(A) It is certain that at least some types of error in protein synthesis are restricted to some unusual codons (see Atkins *et al.*, 1979 for examples).

(B) The protein degradation system is likely to be very efficient in eliminating some errors, but not others. This results in an even higher level of restriction in the location of accumulated (as opposed to synthesized) protein synthesis errors.

(C) According to some error theories, death may occur not because the errors accumulated are too numerous but because the synthesized errors are numerous enough that so much energy is spent degrading faulty proteins that the cell is no longer efficient enough to live.

(D) In *E. coli*, a "misincorporation" error into proteins S12 (Yates *et al.*, 1977; Strigini and Gorini, 1974; Biswas and Gorini, 1972) S17 (Bollen *et al.*, 1975) or L6 (Kuhberger *et al.*, 1979) causes diminution in the number of some translational revertants (in other words, a protein synthesis error can create a *reduction* in the production of some other protein synthesis errors, including itself). By analogy one can imagine that through increased errors in protein synthesis in the host, the number of viral revertants may increase, decrease or stay the same.

Consistent with this apparently paradoxical prediction of the error theories of aging are the results of Fulder (1977). He proposed to test whether the observation of Holland *et al.* (1973) could be generalized, at least within the cultured human fibroblast system. He infected late- and middle-passage human fibroblasts with three temperature-sensitive mutants of Herpes virus. He found a variety of results. The mutant most dependent on host enzyme for its replication (tsE) showed a tenfold increase in reversion rate (in apparent contradiction to the results of Holland *et al.*, 1973). On the other hand, another mutant (tsG) experienced a hundredfold *decrease* in reversion rate (one should note that the reversion rate of tsG in young fibroblasts was aberrantly high (10^{-3}-10^{-4}), suggesting that tsG is an unusual mutation).

(E) Holland *et al.* (1973) did not necessarily study old fibroblasts. Fibroblast sterility is known to precede death by many months (Matsumura *et al.*, 1979). In this review, we will use the 1979 terminology of Matsumura, Zerrudo and Hayflick. Thus cells at

their terminal passage are called presenescent. The long period which follows the last passage and precedes death is called senescence. To distinguish late-passage cells from terminal-passage cells (or terminal phase III cells), we will call the former cells "early presenescent." The new terminology emphasizes the need to study old cells and that sterility (or loss of replicative capacity) is not a synonym of death (Kirkwood, 1980). (Previously, it was common practice to call late passage fibroblasts "senescent" and loss of replicative capacity "death.") Accordingly, Holland *et al.*, (1973) studied presenescent fibroblasts.

Using crude methods (see Fulder, 1977, for some arguments) such as thermal inactivation, analysis of viral proteins by gel electrophoresis, and virus yields, two other groups were unable to detect alterations in viruses made by presenescent cultured fibroblasts (Pitha, Adams and Pitha, 1975; Pitha, Stork and Wimmer, 1975; Tomkins *et al.*, 1974) or by fibroblasts isolated from mature human beings (Danner *et al.*, 1978). Such negative results, as obtained, are compatible with error theories. For clarifications, see, for example 2(E).

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2. *Abnormalities of macromolecules.* A number of studies have been carried out to determine if there is evidence of age-related change in the molecular characteristics of DNA, RNA or proteins, on the assumption that such changes would indicate alterations or errors in synthesis.

(A) Goldstein and Csullog (1977) compared macromolecular synthesis in human fibroblasts at 42°C and 37°C. Their rationale was that if errors were introduced into one or more enzymes involved in macromolecular synthesis, these might be detectable as a temperature dependence (the authors used, to my understanding, incorrectly, the terms heat lability, or sensitivity, to describe temperature dependence) of the rate of incorporation of labeled precursors into DNA, RNA or protein. The authors did not detect increased temperature dependence as the cells progressed from phase II to early phase III (13 population doublings from sterility). In mid phase III (2 population doublings from sterility), they did detect increased "heat lability."

It is concluded that the onset of cellular aging *in vitro* precedes any detectable increase in "heat liability" of synthesis of the three classes of macromolecules. While this work does not support the error theories, I do not believe (nor did the authors) it offers strong evidence against such theories. (a) The authors discuss a number of possible explanations for the failure to find increased defective biosynthesis of macromolecules, e.g. increased proteolysis of defective molecules, insensitivity of their method, etc. (b) To measure a temperature dependence in the metabolism of a group of enzymes is not necessarily to measure a "heat-lability"in these enzymes. Perhaps this is why the "heat inactivation" curves measured by the authors had shapes at variance with what is usually expected, i.e. a linear combination of exponential declines. (c) Error theories are meant to explain mortality, not all the changes which accompany aging. It may well be that wear and tear or genetic programming are better explanations of many such changes.

(B) Pendergrass *et al.* (1976) examined presenescent human skin fibroblasts for the appearance of abnormal glucose-6-phosphate dehydrogenase (G-6-PD). They looked both for the appearance of thermolabile G-6-PD and for an increase in immunologi-

cally intact but enzymatically inactive G-6-PD. Less than 10% thermosensitivity and no change in immunotitration accompanied presenescence. In similar experiments, Holliday and Tarrant (1972) reported that 20% of the enzyme was thermolabile in late passage cells; this may simply be due to a shift in equilibrium favoring the more heat sensitive (tetrameric) form of the enzyme (Duncan *et al.*, 1977). As Pendergrass *et al.* (1976) point out, failure to replicate may be the cell's response to apparently negligible increases in translation error. Thereafter, the errors may increase exponentially until they cause death.

Alternatively, it is conceivable that lack of replicative capacity is a developmental event (see Kontermann and Bayreuther, 1979; Martin *et al.*, 1975, for evidence), whereas cell death, which occurs many months later, is due to error catastrophe (Evans, 1979). It is obvious that the experiments of Pendergrass *et al.*, (1976) can lead to many and divergent interpretations. They do, however, indicate that a large number of thermolabile proteins need not accompany presenescence.

(C) Numerous other studies have been done on the specific activity and thermolability of enzymes, quantities which reflect the quality of the protein, as a function of age. Of the 34 source-papers I could read on this subject, 25 detected alterations in 11 different proteins belonging to organisms as varied as old fibroblasts, *Neurospora*, rats, nematodes, mice, rabbits, humans. On the other hand, 11 papers could not detect alterations in 11 different proteins (not necessarily the same as in the above studies) from five different old or adult organisms. I can make no generalization out of these 34 papers. For detailed criticisms of the experiments on altered and non-altered proteins as a function of age, consult Gershon and Gershon (1976), Gershon (1979), Holliday (1975), Rothstein (1979) and Kirkwood and Holliday (1975).

(D) Errors in protein synthesis could yield altered DNA polymerases which should increase the mutation rate in all the genes including those for DNA and RNA polymerase. The end result can be an exponential increase in mutations. Fulder (1979) and Fulder and Holliday (1975) attempted to measure the frequency of mutation during the presenescence of cultured human fibroblasts using a system which detects a variant form of glucose-6-phosphate dehydrogenase in both viable and nonviable cells. They observed a rapid rise (approximately exponential) in cell variants during the last 10 passages. However they could not prove that the variants were indeed mutants. Using apparently better understood markers, Gupta (1980) could not detect any exponential increase in mutational events during the first 70% of the cellular replicative life-span.

The fidelity of partialy purified DNA polymerase isolated from cultured human fibroblast has been studied as a function of cell generation (Murray and Holliday, 1981; Linn *et al.*, 1976). In old cells, the DNA polymerase activity made two to five times more polymerization errors, depending on which template and which DNA polymerase fractions were used to direct synthesis. *In vivo*, but limited, analyses have been performed on other cell types: Barton *et al.* (1974) have reported a twofold decrease in the fidelity of DNA polymerase isolated from aged mouse liver, while Agarwal *et al.* (1978) could detect no increase in the error frequency of DNA polymerase α isolated from lymphocytes of old people.

(E) Bradley *et al.* (1975) reported a test of the error theory of aging by measuring the susceptibility to proteolysis of soluble proteins extracted from cultured WI 38 cells in phase III. Their rationale is that the rate at which a protein is enzymatically degraded is a function of its secondary and tertiary structures, which are themselves functions of the primary structure. If the correct amino acid sequence is altered through mistranslation then the protein may no longer be in its native conformation and so may be more susceptible to proteolysis because of the exposure of labile peptide bonds.

The authors checked the sensitivity of their technique in the following ways. (1) They iodinated different average numbers of tyrosine residues per average protein molecule in a WI 38 extract. An average of 4×10^{-3} iodine/amino acid is sufficient to increase the proteolytic rates by a detectable amount. The authors stress that these values are only estimates of the mean amount of iodination for an average protein molecule in extracts of heterogeneous proteins. Some proteins, having no tyrosine and therefore not easily iodinated, artificially lower the average value. Therefore, it is likely that only proteins binding more than 4×10^{-3} iodine/amino acid (i.e. for example those with a high tyrosine content) are made more susceptible to proteolysis. It seems fair to say that Bradley's method is likely to be able to select out a protein containing about 10^{-2} iodine/amino acid. (2) They showed that proteins synthesized during 1.25 h in medium containing canavanine are degraded by pronase more rapidly than are proteins synthesized during 1.25 h in medium containing arginine. Evaluation of the number of canavanine (analogues of arginine) incorporated per synthesized protein is subject to at least two assumptions, one regarding the final specific activity of ^{14}C-canavanine and one regarding the proportion of the total protein which will have been synthesized. It seems reasonable to attach a 50% uncertainty to the estimates derived from each respective assumption. This leads us to conclude that the authors are likely to be able to detect protein containing 2×10^{-2} canavanine/amino acid. Whether this means that they are able to detect a protein containing 2×10^{-2} misincorporated natural amino acids/amino acid is quite another story and requires a final assumption: that exogenous protease detects misincorporated natural amino acids as easily as it detects amino acid analogs. We now review the results.

The authors could not consistently detect increased proteolytic susceptibility before terminal phase III (i.e. early presenescence). They *did* detect increased proteolytic susceptibility by the time the cultures had reached their last population doubling (i.e. presenescence), and did not exclude the possibility that a gradual increase in altered protein occurred during early presenescence that was below the sensitivity of their assay. Numerically, the data mean that before fibroblasts reach terminal doubling level, the error frequency during protein synthesis is likely to be less than 2×10^{-2} error/amino acid. At the last passage, which itself may precede cell death by many months, the data are consistent with error frequencies larger than 2×10^{-2}/amino acid. Since the normal misincorporation frequency is $\sim 10^{-4}$ the results are quite compatible with error theories. The authors suggested that if increased proteolytic susceptibility was a function of increased mistranslation, then one interpretation of the data was that mistranslation occured mainly at the very end of phase III. Such a statement argues neither in favor of nor against error theories. The authors also suggested that since other "senescent" cellular changes were detectable before terminal phase III, mistranslation would more likely be an effect rather than a cause of *in vitro*

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"senescence". This statement is valid if (1) by "senescence" it is meant sterility and if (2) mistranslation starts occurring long after replicative capacity has been lost. To have an impact on error theories the authors would then have to demonstrate that fibroblast sterility is a late symptom of senescence. Such is not *a priori* obvious. (See Evans, 1979, for many arguments that senescence starts after a cell has become sterile.)

In my opinion, the authors' data are damaging to the error theories of fibroblast aging only if it can be shown that inducing an error content of more than 2×10^{-2} errors/amino acid does not produce death in a fibroblast and if proteases do detect a protein having a wrong natural amino acid as easily as they would a protein having an analog such as canavanine.

3. Misincorporation of amino acids or amino acid analogs. As a direct assessment of translation errors, several investigators have measured the rate of misincorporation into proteins of known composition, and determined whether or not the error rate increases with age.

Mariotti and Ruscitto (1977) studied protein synthesis fidelity as a function of age using a rat liver cell-free system. They claimed that 12-month-old rats misincorporate leucine (under poly U programming) two to five times more than 3-month-old rats.

Yang *et al.* (1977) found that ribosomes from adult and embryonic chick brain tissues have similar fidelities in the translation of poly U. This is not surprising and cannot be considered as an argument for or against error theories. Mori *et al.* (1979) studied mouse liver ribosomes *in vitro*. The results are scattered, consistent with anything between no change in fidelity and a fivefold decrease in accuracy with age. It should be noted that the ribosomes they used were about 10 times less active than normal (Laughrea, 1981a) and that very old animals were not studied. Kurtz (1975) did study old animals but, unexpectedly, observed a slight fidelity increase with age in a mouse liver system. This result can be explained away as a starvation artefact (Laughrea, 1981a) or it can be criticized because inactive and very error-prone ribosomes were used in the experiments (Laughrea, 1981a, 1981b).

Translation of poly U by cell-free extracts of cultured human fibroblasts revealed no change in accuracy between young and early presenescent (late passage) cells (Buchanan *et al.*, 1980; Wojtyk and Goldstein, 1980). While these results do not support error theories of aging, they do not refute them either because (1) the experimental set up is critically dependent on the presumption that the eventual steriiity of cultured fibroblasts is an adequate model of animal aging, (2) only one type of mistranslation has been studied, and (3) the *in vitro* assay did not necessarily reflect the *in vivo* error levels.

Cultured fibroblasts of different ages have also been used to perform an *in vivo* measurement of translation errors (Harley *et al.*, 1980). The measurements needed to be made under starvation conditions; it remains to be seen whether the results can be extrapolated back to normal growth conditions (Gallant and Foley, 1980). Only one type of error has been studied: the replacement of histidine by neutral amino acids. While the conclusions from this and other papers may eventually prove correct, the data base remains insufficient and more work is needed in order to refute or confute the error theories of aging (Kirkwood, 1980).

Indirect experiments, using amino acid analogs, can give some hints about the quantitation of error increase with age. The methionine/ethionine incorporation ratio into pro-

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teins of adult mouse or middle passage human fibroblasts is about 100/1 (Ogrodnik *et al.*, 1975; Lewis and Tarrant, 1972). In late passage human fibroblasts, this ratio is down to 10/1 (Lewis and Tarrant, 1972) suggesting that presenescence is accompanied by a tenfold or so increase in translation errors. Ethionine misincorporation has also been studied in ribosomes from adult mouse liver (i.e. 495 days old while the mean life-span was 750 days). The results are complex (Ogrodnik *et al.*, 1975); some ribosomal proteins showed no increase in ethionine incorporation (relative to such proteins isolated from 115-day-old animals), while others showed a 90% increase in ethionine incorporation. From the point of view of error theories, it is important to know the error rates in *old* mice, i.e. those whose age approximates the life-span of the species. As long as one is studying a phenomenon which is not expected to increase exponentially with time, the study of the adult, as opposed to the old, is justifiable. However when the parameter under study may increase exponentially with age, a negative result in adult animals does not exclude a positive result in animals that are close to the *maximum* life-span of their species. The following example is an illustration of this point.

Johnson and Erner (1972) studied changes in neuron number as a function of aging in mice. They could not detect any change until the mice were 24 months old (their mean life-span was 16 months). Had they stopped there they could have concluded that neuron mortality has nothing to do with aging. Instead, they continued to get data until the mice were 29 months old. Such apparently senescent mice (99% of their cohort had already died) had lost 65% of their neurons. Data collected over the whole range of age enabled them to show a possible exponential decrease in neuron survival with age. Furthermore, a Gompertz plot of their data (log of the rate of death as a function of age) indicated that the rate of dying of the neurons was similar to the rate of aging of the species. This suggests that neuron dying is the direct cause for senescent death in that mouse species. It would be interesting to know more about brain or neuron protein synthesis accuracy as a function of age.

Like mouse neuron mortality, translation errors may become detectable only when animals are far older than the mean life-span for their species. Consequently, the negative results of several other reports, based on study of animals clearly younger than the average life-span (Reis, 1976; Hirsch *et al.*, 1976) may have little significance.

Popp *et al.* (1976) studied the misincorporation of isoleucine (human hemoglobins A and A_2 contain no ileu) into human hemoglobin isolated from Marshall Islanders aged 20–60. Their results are graphed in Figure 1 (the inter- and extrapolated lines are mine). Clearly the results are inconclusive and may be seen as consistent with either no change or, according to the authors, with a 50–100% increase in infidelity between the ages of 20 and 50.

In a related experiment, Buchanan and Stevens (1978) attempted to measure methionine misincorporation into H1 histone from cultured fetal lung fibroblasts (H1 histone normally contains no methionine). The methionine incorporation into that protein was too low for them to measure. They could, of course, put upper limits to the error frequencies: the error level for the misincorporation of methionine into H1 histone was less than 7×10^{-5} met/aa and 30×10^{-5} met/aa in early and late passage cells, respectively.

Can premature senescence be caused by artificial increase in translation errors?

Incorporation of amino acids or nucleoside analogs has been used as a provocative test

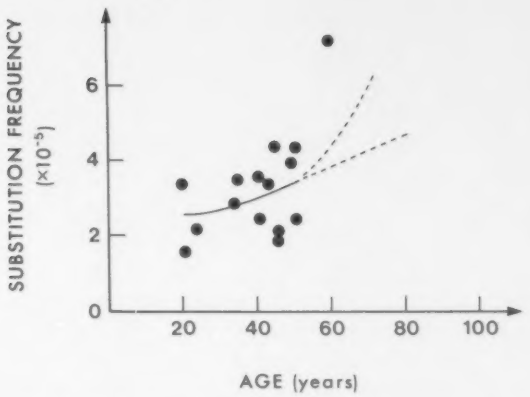

FIG. 1. The misincorporation of isoleucine into human hemoglobin isolated from Marshall Islanders aged 20–60.

of the error theories of aging. The rationale is that if an exponential increase in translation errors is the cause of senescent death, artificially increasing translation errors may cause premature senescence and a reduction of the maximum life-span.

Growth of fibroblasts in 5-fluorouracil may (Holliday and Tarrant, 1972) or may not (Lewis, 1976) reduce replicative life-span depending on the experimental conditions used. Using amino acid analogs, it is possible to reduce (Harrison and Holliday, 1967) or leave unchanged (Reis, 1976; Harrison and Holliday, 1967; Bozcuk, 1976) the life-span of *Drosophila*, depending on the experimental conditions. When fed to *Podospora* amino acid analogs have complex effects which can be interpreted as reduced life-span (Holliday, 1969). Administration of amino acid analogs to the mouse may reduce longevity (Holliday and Stevens, 1978).

Ryan *et al.* (1974) grew human fibroblasts in the presence of amino acid analogs. Because they used radioactive analogs, interesting data can be extracted from their paper. Assuming that (1) 1 ml of cell extract contains at least 1 mg of protein and (2) 3% of the total protein content got synthesized during the 45 min pulse (Bradley *et al.*, 1975), the numbers in their Tables 1 and 2 indicate that growth in the presence of 0.9 μM ethionine and 50 μM p-fluorophenylalanine (p-FPA) yields a translation error rate of 1.95×10^{-2}/aa, 99% of the errors being contributed by p-FPA. Growth in the presence of 9.2 μM ethionine and 17.2 μM p-FPA sterilized the cells within five population doublings (they should normally have grown for another 15 population doublings). This suggests that the cell cannot stand error rates of the order of 7×10^{-3} per amino acid.

In apparent agreement with the error theories of aging, Ryan *et al.* (1974) found that a 30-day pulse of analogs produced premature sterility. In apparent contradiction, the cells were able to recover from a 7-day pulse. For an explanation of this last phenomenon, see Lewis (1976). A short pulse may represent a shock and lead to derepression of the degradation enzymes, etc. In real life there is probably no error shock, only a gradual increase which would be insiduous enough to prevent generation of cellular defense mechanisms. Both opponents (Lints, 1978, p. 26) and proponents (Holliday and Stevens, 1978) of the error theories think that experiments with amino acid or base analogs cannot be regarded

as an adequate test of the error theories of aging because analogs aren't part of the normal cellular life.

Do enzyme activities or numbers decrease with aging?

Numerous studies on tissues taken from whole animals have shown that the total activity of many enzymes change with age. These studies have not led to any important generalization (Wilson, 1973; Finch, 1972). The activities of some enzymes go down with age, but many enzyme activities are unaffected and some increase. The changes do not appear to be a function of the class of enzyme.

Miquel and Johnson (1979) provided evidence for ribosomal loss associated with the senescent deterioration. Their study is purely descriptive and makes no attempt at explaining what ribosome loss may mean. There is a relation between the growth rate of *E. coli* and the number of ribosomes per cell (Kjelgaard and Gaussing, 1974; Maaløe and Kjeldgaard, 1966). It is also known that the intracellular concentration of ribosomes is lower in resting Syrian hamster cells than in proliferating cells (Becker *et al.*, 1971) (see also Darzynkiewicz *et al.*, 1979). Presumably, the growth rate of many old cells is lower (or their metabolic activity if they are not dividing cells), hence the lower concentration of ribosomes. The authors report many observations of normal ribosome levels for example in old brain tissue. This suggests that the metabolic activity of these tissues did not go down significantly with age.

How much translational error is necessary to cause cell death?

It appears that a good way to argue against (or for) the errors theories is to ask how much translational ambiguity is necessary to kill a cell, and show that a cell can (or cannot) survive high levels of ambiguity without dying. Edelman and Gallant (1977) showed that when *E. coli* is grown in the presence of streptomycin (a misreading-inducing antibiotic), misincorporation of cysteine into flagellin can be as high as 7.5×10^{-5} cys/aa without being lethal to the cell. Assuming that this measured misincorporation rate applies to the other 19 amino acids, then the results of Edelman and Gallant (1977) suggest that *E. coli* can support error rates of the order of 1.5×10^{-3}/aa into flagellin without dying (see also Gallant and Palmer, 1979).

The experiments of Gallant and collaborators are important but they may be misleading because they focused on a non-essential protein. The error rates reported may have killed the protein without killing the cells (the authors did not check, to my knowledge, whether the bacterium was still motile after the drug treatment). Ogrodnik *et al.* (1975), O'Farrell (1978) and Parker *et al.* (1978) have presented evidence that different proteins accumulate a particular translation error at widely different rates. It is possible that because flagellin is relatively insignificant for cell survival, the cell can tolerate a much higher error rate in this protein than in its vital proteins.

Amino acid analogs have also been incorporated into proteins (Taub, 1977; Fowder *et al.*, 1967; Shive and Skinner, 1963; Barker and Burton, 1979; Beaud and Dru, 1980). Such experiments are not relevant for obvious reasons: a misincorporation error consists of the insertion of an amino acid which generally bears little resemblance to the one it replaces. Because, by definition, analogs have structures very similar to what they replace, their incorporation must generally have milder effects than incorporation of another natural amino acid. This is why incorporation rates of 0.5 analog/aa are compatible with life

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(Moore, 1979) (the analogs were deuterated amino acids). On the other hand, incorporation of 5×10^{-3} 5-fluorotryptophan/aa may be sufficient to prevent growth of, respectively, *E. coli* (Pardee et al., 1956; Pardee and Prestidge, 1958) or A9 fibroblasts (Taub, 1977).

Amino acid starvation could become a useful tool to ask how much translation ambiguity is necessary to kill a cell (O'Farrell, 1978; Parker *et al.*, 1978; Parker and Friesen, 1980), as long as the starvation is not so extreme that protein degradation activity become similar, in magnitude, to protein synthesis activity (Stanners *et al.*, 1978; Stanners and Thompson, 1974), and as long as it can be shown that many errors at a few codons yield the same physiological effects as twentyfold fewer errors (there are 20 amino acids involved in translation; starvation measurements usually involve starvation for only one amino acid, i.e. translation errors at a few specific codons) distributed over all codons.

CONCLUSION

Present experimental evidence suggests (or is consistent with the idea) that aging is accompanied by at least a modest increase in error frequency during information transfer from nucleic acids to protein. However, it is not clear whether or not this putative increase is highly significant as a cause of aging or senescent death.

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Experimental Gerontology, Vol. 17, pp. 319–320, 1982
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BOOK REVIEW

LIFE EXTENDING TECHNOLOGIES—A TECHNOLOGY ASSESSMENT

Edited by THEODORE J. GORDON, HERBERT GERJUOY, and MARK ANDERSON
1980, Pergamon Press, New York. ISBN: 0-08-0231322. $33.00

REVIEWER: WARD DEAN,* U.S. Army, Taegu, Korea

THE STATED purpose of this book was to make an assessment of the prospects for developing successful life-extending techniques by the year 2000, and to determine the social, political and economic effects these advances would have on society. Despite the distinguished editorship and generally high caliber of individuals whose opinions were solicited, the book was a disappointment to this reviewer.

The book was based on the results of an interview system, by which an advisory panel of experts were interviewed concerning their assessment of the chances for successful technological developments within their particular area of expertise to take place before the year 2000. The results of the first round of interviews were then compiled, and the results were mailed to the respondents. This was followed up by a telephone interview, the replies to which formed the basis for a calculated statistical probability of certain events occurring before the year 2000. Although anonymity was guaranteed to the respondents, this did not contribute to rash overstatement, as the general consensus was that life-extending technologies would not be available until 1990 or after.

The Introduction points out that there are generally two classes of life-extending technologies—those which increase the likelihood that an individual will survive to complete the life span ("curve squaring"), and those which extend the span. It was also noted that there are three basic approaches to life-extension—Immortalist, Incrementalist, and Meliorist. The Immortalists argue that the prospect of substantially extending both life expectancy and life span will depend on the ability to counteract or reverse the basic primary aging processes, and that it will not be necessary to seek to eliminate the diseases of old age if persons can be kept from getting old. The Incrementalists are more traditionally oriented, in favor of the step-by-step attack on individual diseases—while the Meliorists

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are more interested in improving the living accommodations of the elderly, and merely making them more comfortable in their remaining years.

The first chapter, Principal Findings, summarized the findings of the group, listing the expected life-extending technologies which they felt were most likely to be available by the year 2000. Also discussed were the economic and social impacts of life extension.

Chapter 2, Biomedical Technologies: State of the Art and Forecasts, is a compilation of currently held theories of aging, and an assessment of their success in contributing to our overall understanding and ultimate control of the aging process. Nothing new in this area was presented in this chapter, and this subject has been covered more completely in other recent books. Also in this chapter were the calculated probabilities of the success of various life-extending technologies in extending life spans by the year 2000. Recommendations for further research directions were also presented—probably the most interesting of which was the recommendation by a small but significant number of experts who felt that undirected basic research was more profitable in the long run than targeted research. This group felt that serendipitous research discoveries would provide breakthroughs of greater significance than would programmed, directed research. They felt that because of the importance of serendipitous discoveries, it was not only impossible to do targeted research, but also that credible forecasts of technological breakthroughs would be equally impossible.

The remainder of the book was concerned with the social, sociological, demographic and economic effects of life extension.

Although policy planners and social gerontologists would probably find this book of some interest, I feel that biological gerontologists could spend their time more profitably in other pursuits.

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Experimental Gerontology, Vol. 17, pp. 321–331, 1982
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CHANGES WITH AGE IN THE HUMAN KIDNEY

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(*Received* 18 *November* 1981)

Abstract—Histologic and micrometric studies have been performed to examine changes with age in the kidney of 100 autopsy cases ranging from 1 to 70 years of age. The number of tubular cells and glomerular cells decreased significantly during aging. The number of glomerular tufts per unit area also decreased significantly with advancing age. The size of malpighian corpuscles, glomerular tufts, nuclei of glomerular cells and nuclei of tubular cells increased significantly in senile kidneys. The statistical analysis revealed that the values of the coefficient of variation generally increased with advancing age.

INTRODUCTION

OF ALL the organs, the kidney seems to have provided the least definite data in relation to changes with age. Tauchi *et al.* (1958) examined the kidneys of Japanese and noted a decrease in the number of renal parenchymal cells. Tauchi *et al.* (1971) examined the kidneys of Japanese and Caucasians (Americans) and reported a decrease in the number of parenchymal cells and tendency to increase in the size of cells as the main characteristics of senile changes. The size of glomeruli did not show any marked change with age in Caucasians, whereas in Japanese a significant decrease in the size of glomeruli was reported with increasing age. Sato *et al.* (1975) observed a decrease in the number of glomeruli/unit volume and increase in the radii of glomeruli in mouse kidney up to 50 weeks of age. The present study has been made to clarify further the mechanism of senile changes of the kidney in Indian autopsy cases by performing histologic and micrometric studies.

MATERIALS AND METHODS

The kidneys of 100 autopsy cases (only accidental cases) from 1 to 70 years of age were used in the present investigation. The human post-mortem material was collected from the police-morgue of N.R.S. Medical College, Calcutta, India. Every care was taken to get the material from healthy normal persons. The kidney tissue was fixed in 10% neutral buffered formalin within 24 hours after death. Paraffin sections were cut at 4 μ and stained with haematoxylin and eosin.

The number of tubular cells and glomerular tufts was counted per unit area. The number of cells in glomerular tufts was counted and the average was taken by examining randomly ten such glomeruli from a single slide. The size of glomerular tufts, the malpighian corpuscles, the nuclei of glomerular and tubular cells was measured by drawing randomly twenty camera lucida diagrams from a single slide on mm² graph paper. The diagrams of glomerular tufts and malpighian corpuscles were drawn under low power field and of nuclei under immersion oil field of the microscope. The size was calculated by taking the square root of the area.

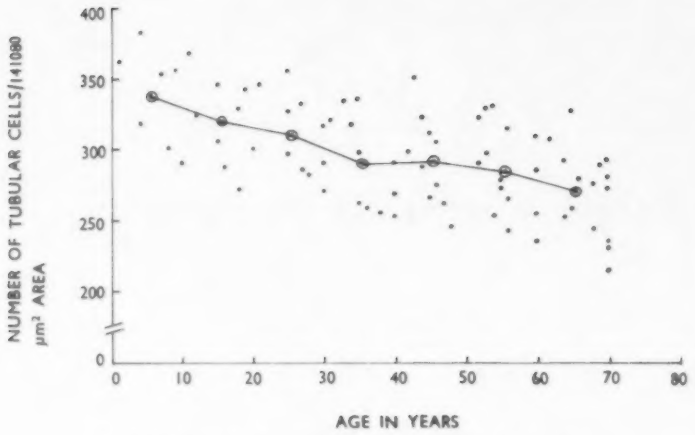

FIG. 1. Changes in the number of tubular cells with aging.

OBSERVATIONS

The present histologic and micrometric studies showed the following changes with age in the human kidney:

Number of tubular cells

The number of convoluted tubular cells per unit area was found to decrease with increasing age (Table 1; Fig. 1). Statistical analysis revealed significant difference in the

TABLE 1. CHANGES IN THE NUMBER OF TUBULAR CELLS IN RELATION TO AGE

Age Group	Age in Years	Number of subjects	Number of tubular cells/141080 μm^2 area
A	1-10	7	337.57 ± 13.19 CV 10.35%
B	11-20	9	319.33 ± 10.18 CV 9.56%
C	21-30	10	309.90 ± 9.21 CV 9.39%
D	31-40	11	290.36 ± 9.89 CV 11.31%
E	41-50	10	292.20 ± 9.89 CV 10.70%
F	51-60	15	283.53 ± 8.01 CV 10.93%
G	61-70	15	269.67 ± 10.64 CV 11.32%

$P < 0.001$ = A and G.
$P < 0.01$ = A and F, B and F, B and G, C and G.
$P < 0.05$ = A and D, A and E, C and F, D and G, E and G.
NS = A and B, A and C, B and C, B and D, B and E, C and D, C and E, D and E, D and F, E and F, F and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

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TABLE 2. CHANGES IN THE NUMBER OF GLOMERULAR TUFTS WITH ADVANCING AGE

Age Group	Age in Years	Number of subjects	Number of glomerular tufts per 14306625 μm^2 area
A	1–10	7	74.86 ± 3.69 CV 13.08%
B	11–20	9	54.11 ± 2.30 CV 12.73%
C	21–30	11	49.18 ± 2.31 CV 15.62%
D	31–40	12	44.83 ± 1.95 CV 15.01%
E	41–50	10	43.70 ± 2.01 CV 14.51%
F	51–60	15	41.27 ± 1.86 CV 17.39%
G	61–70	15	39.07 ± 1.81 CV 17.96%

$P < 0.001$ = A and B, A and C, A and D, A and E, A and F, A and G, B and F, B and G.
$P < 0.01$ = B and D, B and E, C and G.
$P < 0.05$ = C and F, D and G.
NS = B and C, C and D, C and E, D and E, D and F, E and F, E and G, C and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

number of tubular cells between the first and the seventh decade (<0.001). There was a tendency to increase in the coefficient of variation with the advancement of age (Table 1).

Number of glomerular tufts

A progressive decrease in the number of glomerular tufts per unit area was noted with increasing age (Table 2; Fig. 2). The difference in the number of glomerular tufts between the first and second decade onwards was significant (<0.001). There was an increase in the coefficient of variation in old individuals (Table 2).

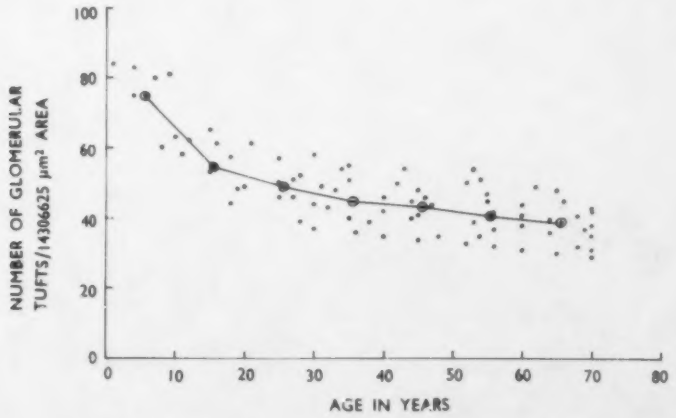

FIG. 2. Changes in the number of glomerular tufts with aging.

TABLE 3. CHANGES WITH AGE IN THE NUMBER OF GLOMERULAR CELLS

Age Group	Age in Years	Number of subjects	Number of cells in glomerular tufts
A	1–10	7	163.29 ± 10.10 CV 16.37%
B	11–20	9	155.78 ± 7.10 CV 13.69%
C	21–30	11	153.90 ± 7.16 CV 15.42%
D	31–40	12	144.67 ± 7.20 CV 17.23%
E	41–50	10	136.80 ± 6.55 CV 15.12%
F	51–60	15	130.93 ± 5.22 CV 15.41%
G	61–70	15	122.53 ± 6.08 CV 19.20%

$P < 0.01$ = A and F, A and G, B and F, B and G, C and G.
$P < 0.05$ = C and F, D and G.
NS = A and B, A and C, A and D, A and E, B and C, B and D, B and E, C and D, C and E, D and E, D and F, E and F, E and G, F and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Number of cells in glomerular tufts

The number of cells in glomerular tufts decreased progressively with advancing age (Table 3; Figs. 3,4,5,6,7). Statistical significant differences were observed between the first and the sixth decade, the first and the seventh decade, the second and the sixth

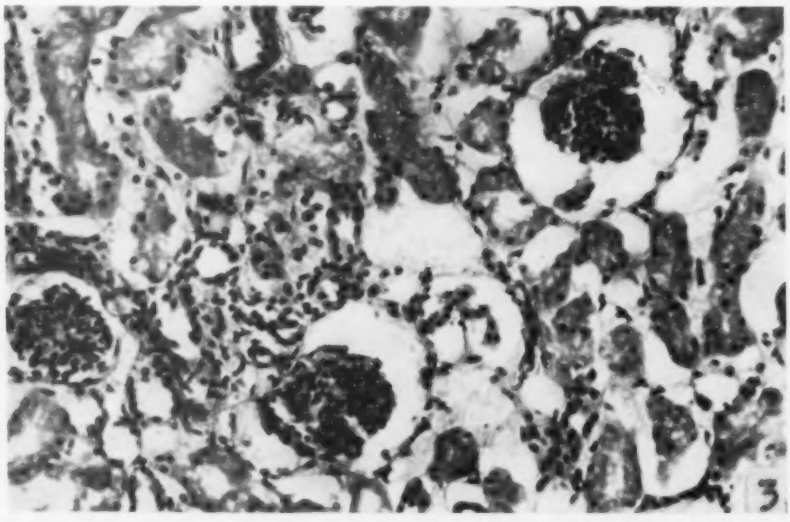

FIG. 3. Age 1 year. Photomicrograph shows the size of glomerular tufts, malpighian corpuscles and the number of glomerular cells. Haematoxylin-eosin × 300.

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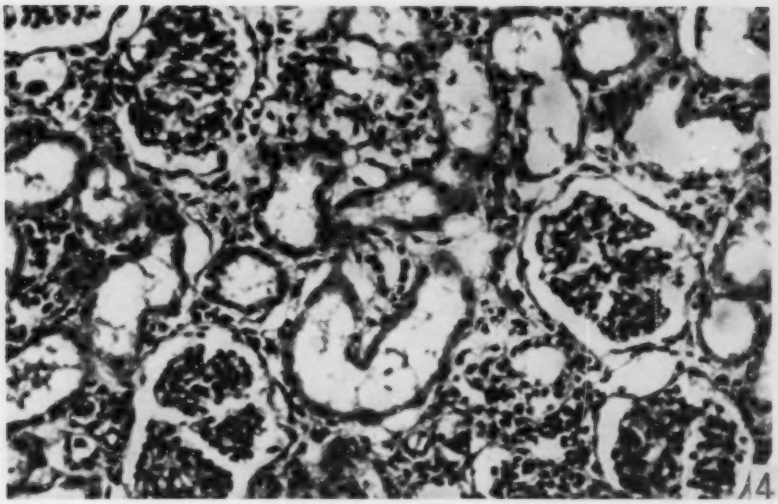

FIG. 4. Age 9 years. Photomicrograph shows the size of glomerular tufts and malpighian corpuscles, and the number of glomerular cells. Haematoxylin-eosin × 300.

decade, the second and the seventh decade, and the third and the seventh decade (<0.01). The coefficient of variation tended to increase with age (Table 3).

Size of the malpighian corpuscles

The size of the malpighian corpuscles was observed to increase with advancing age (Table 4; Figs. 3,4,5,6,8). The differences in size of the malpighian corpuscles between the

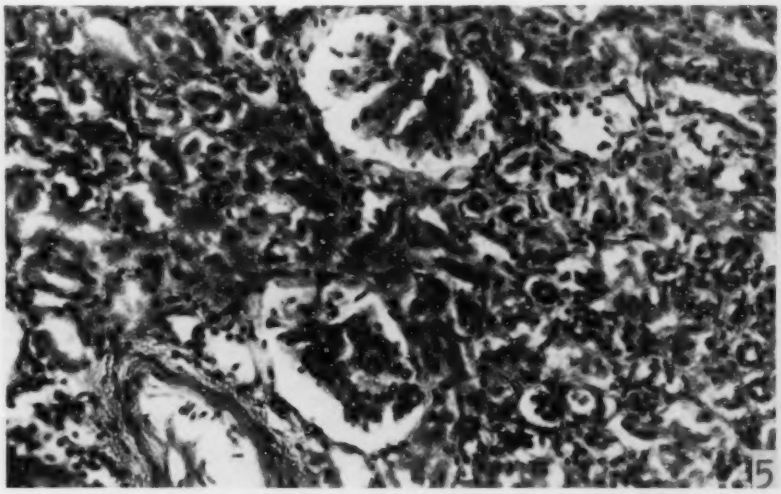

FIG. 5. Age 40 years. Photomicrograph shows an increase in the size of the glomerular tufts and the malpighian corpuscles, and a decrease in the number of glomerular cells in comparison to those of 1-year and 9-year-old individuals (Figs. 3 & 4). Haematoxylin-eosin × 300.

FIG. 6. Age 70 years. Photomicrograph shows an increase in the size of glomerular tufts and malpighian corpuscles, and a decrease in the number of glomerular cells in comparison to those of 1.9 and 40-year-old individuals (Figs. 3, 4, & 5). Haematoxylin-eosin × 300.

first decade and second decade onwards were signficant (<0.001). The coefficient of variation tended to increase with age (Table 4).

Size of the glomerular tufts

The size of the glomerular tufts increased progressively with advancing age (Table 5; Figs. 3,4,5,6,9). The increased differences in the size of glomerular tufts between the first decade and second decade onwards were significant (<0.001). The coefficient of variation was observed to increase in old individuals (Table 5).

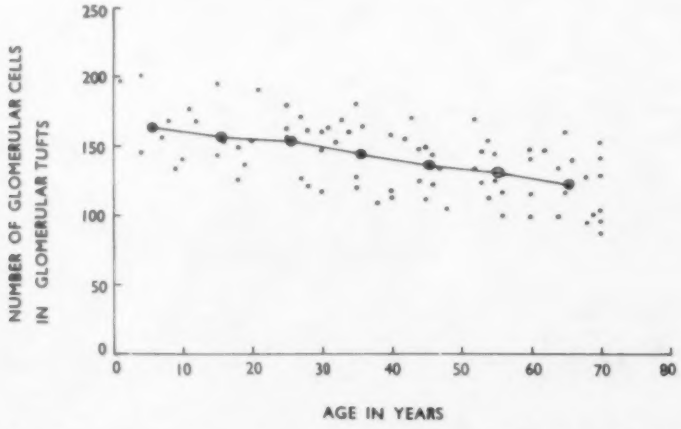

FIG. 7. Changes in the number of glomerular cells in glomerular tufts with aging.

TABLE 4. CHANGES WITH AGE IN THE SIZE OF THE MALPIGHIAN CORPUSCLES

Age Group	Age in Years	Number of subjects	Size of the malpighian corpuscles in μm
A	1–10	7	121.04 ± 3.17 CV 6.93%
B	11–20	9	147.86 ± 4.42 CV 8.96%
C	21–30	11	152.72 ± 3.63 CV 7.89%
D	31–40	12	151.52 ± 3.98 CV 9.09%
E	41–50	10	158.31 ± 4.45 CV 8.89%
F	51–60	15	163.95 ± 3.45 CV 8.14%
G	61–70	15	166.89 ± 3.50 CV 8.34%

$P < 0.001$ = A and B, A and C, A and D, A and E, A and F, A and G.
$P < 0.01$ = B and G, D and G.
$P < 0.05$ = B and F, C and F, C and G, D and F.
NS = B and C, B and D, B and E, C and D, C and E, D and E, E and F, E and G, F and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Size of the nuclei of glomerular cells

It was observed that the size of the glomerular cells nuclei increased progressively with age (Table 6; Fig. 10). Statistical analysis revealed significant difference between the first and the seventh decade (<0.001). The coefficient of variation increased with advancing age (Table 6).

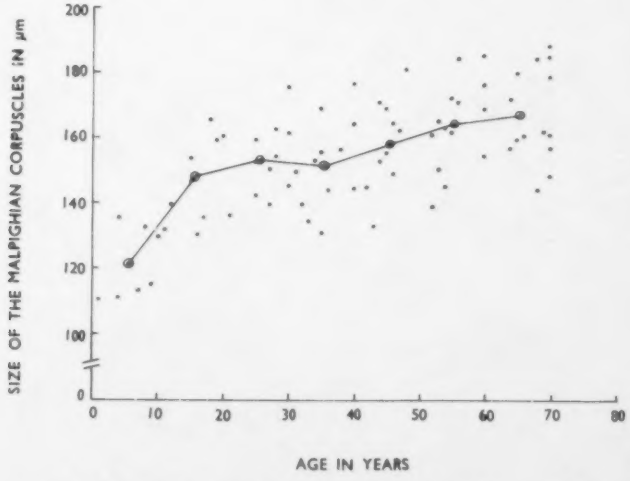

FIG. 8. Changes with age in the size of the malpighian corpuscles.

TABLE 5. CHANGES IN THE SIZE OF THE GLOMERULAR TUFTS IN RELATION TO AGE

Age Group	Age in Years	Number of subjects	Size of the glomerular tufts in μm
A	1–10	7	85.42 ± 2.95 CV 9.14%
B	11–20	9	112.03 ± 3.19 CV 8.54%
C	21–30	11	115.26 ± 2.94 CV 8.46%
D	31–40	12	114.91 ± 2.88 CV 8.69%
E	41–50	10	121.68 ± 3.23 CV 8.40%
F	51–60	15	122.55 ± 2.93 CV 9.25%
G	61–70	15	126.96 ± 3.18 CV 9.70%

$P < 0.001$ = A and B, A and C, A and D, A and E, A and F, A and G.
$P < 0.01$ = B and G.
$P < 0.05$ = B and F, C and G, D and G.
NS = B and C, B and D, B and E, C and D, C and E, C and F, D and E, D and F, E and F, E and G, F and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Size of the nuclei of tubular cells

As shown in Table 7, the size of the nuclei of tubular cells increased progressively with advancing age (Fig. 11). The difference in the size of the nuclei of tubular cells between the first and the seventh decade was significant (<0.001). There was a tendency to increase in the coefficient of variation with increasing age (Table 7).

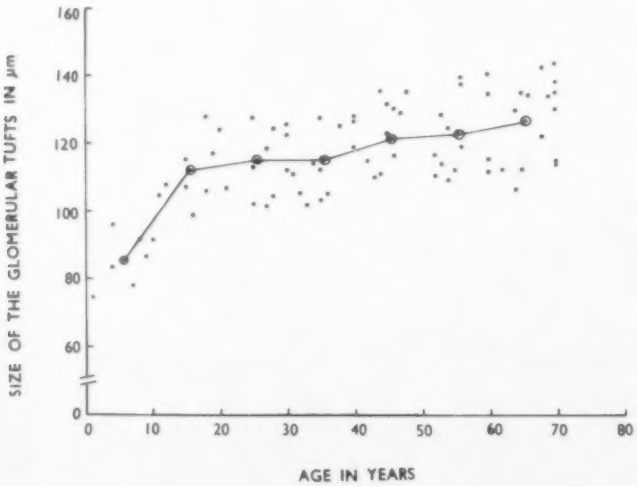

FIG. 9. Changes with age in the size of the glomerular tufts.

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TABLE 6. CHANGES WITH AGE IN THE SIZE OF THE NUCLEI OF GLOMERULAR CELLS

Age Group	Age in Years	Number of subjects	Size of the nuclei of glomerular cells in μm
A	1–10	7	3.84 ± 0.0691 CV 4.76%
B	11–20	9	3.94 ± 0.0649 CV 4.94%
C	21–30	11	3.99 ± 0.0613 CV 5.09%
D	31–40	12	3.97 ± 0.0702 CV 6.13%
E	41–50	10	4.02 ± 0.0935 CV 7.35%
F	51–60	15	4.09 ± 0.0898 CV 8.49%
G	61–70	15	4.16 ± 0.0828 CV 7.70%

$P < 0.01$ = A and G.
$P < 0.05$ = A and F, B and G.
NS = A and B, A and C, A and D, A and E, B and C, B and D, B and E, B and F, C and D, C and E, C and F, C and G, D and E, D and F, D and G, E and F, E and G, F and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

DISCUSSION

The present study revealed a significant decrease in the number of convoluted tubular cells with the advancement of age (Table 1; Fig. 1). Sato *et al.* (1969) and Tauchi *et al.* (1971) also reported a decrease in the number of epithelial cells of convoluted tubules in

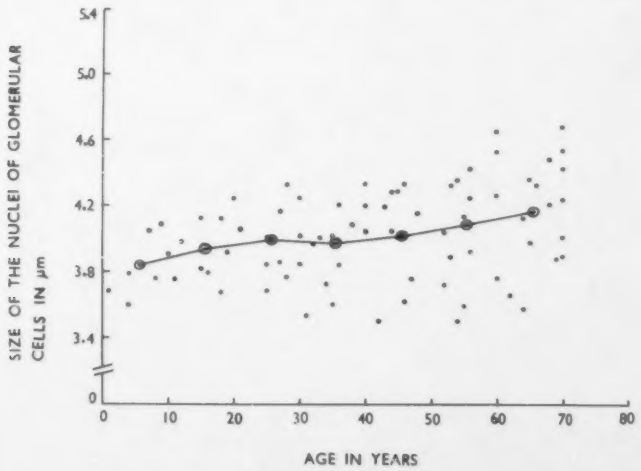

FIG. 10. Changes with age in the size of the nuclei of glomerular cells.

TABLE 7. CHANGES WITH AGE IN THE SIZE OF THE NUCLEI OF TUBULAR CELLS

Age Group	Age in years	Number of subjects	Size of the nuclei of tubular cells in μm.
A	1–10	7	3.95 ± 0.0902 CV 6.05%
B	11–20	9	3.99 ± 0.0847 CV 6.36%
C	21–30	11	4.09 ± 0.0879 CV 7.12%
D	31–40	12	4.16 ± 0.0897 CV 7.47%
E	41–50	10	4.22 ± 0.1111 CV 8.32%
F	51–60	15	4.32 ± 0.0836 CV 7.49%
G	61–70	15	4.48 ± 0.0993 CV 7.52%

$P < 0.001$ = A and G.
$P < 0.01$ = A and F, B and F, B and G.
$P < 0.05$ = C and G, D and G.
NS = A and B, A and C, A and D, A and E, B and C, B and D, B and E, C and D, C and E, C and F, D and E, D and F, E and F, E and G, F and G.

$$\text{CV (Coefficient of Variation)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

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senile human kidneys. Tauchi (1961) considered the decrease in the number of cells due to an extracellular fluid factor-so-called inhibitory factor for cell division.

Arataki (1926) and Moore and Hellman (1930) reported a decrease in the number of glomeruli in rats with increasing age. Sato *et al.* (1975) observed a significant decrease in

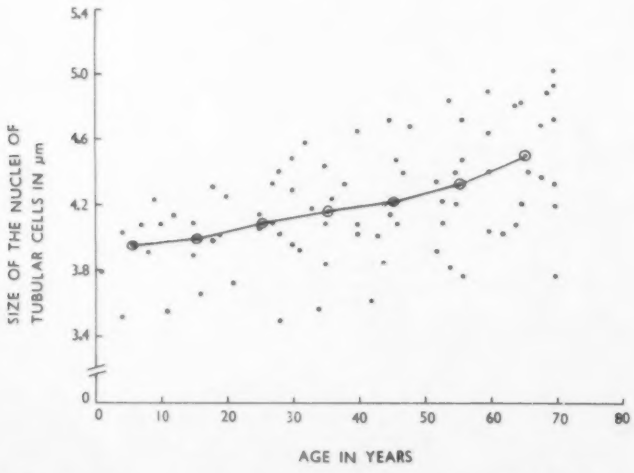

FIG. 11. Changes with age in the size of the nuclei of tubular cells.

the number of glomeruli up to 50 weeks of age in mouse kidney. A decrease in the number of glomeruli was also reported in human beings by Moore (1931) and Korenchevsky (1961). The present study showed a significant decrease in the number of glomeruli with increasing age (Table 2; Fig. 2). The number of glomerular tufts was found to be reduced to nearly about half in the seventh decade in comparison to that of the first decade.

The present study also showed a significant decrease in the number of glomerular cells with increasing age (Table 3; Fig. 7). Tauchi *et al.* (1971) observed a decrease in the number of glomerular cells with age in the kidneys of Japanese and Caucasians (Americans).

The size of malpighian corpuscles was observed to increase with advancing age (Table 4, Fig. 8). A survey of literature indicates that no study has been made so far on the size of malpighian corpuscles in men of different age groups.

The present study revealed a significant increase in the size of glomerular tufts with increasing age (Table 5; Fig. 9). Tauchi *et al.* (1971) reported a significant decrease in the size of glomeruli with age in Japanese. However, they did not observe any significant change with age in Caucasians. Sato *et al.* (1975) showed an increase in size of average radii of glomeruli in mice with advancing age.

The present investigation also indicated a significant increase in the size of the nuclei of glomerular cells with advancing age (Table 6; Fig. 10). This is a pioneer observation of the present study.

An increase in the size of nuclei of tubular cells during aging was observed in the present study (Table 7; Fig. 11). Tauchi *et al.* (1971) also reported a tendency to increase in the size of the nuclei of tubular cells with age in human beings.

The present study showed a significant decrease in the number of tubular cells and glomerular cells with increasing age. The number of glomerular tufts also decreased significantly with advancing age. These changes are followed by an increase in the size of glomerular tufts, malpighian corpuscles, nuclei of glomerular cells and nuclei of tubular cells in senile kidneys.

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Experimental Gerontology, Vol. 17, pp. 333–341, 1982
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0531-5565/82/050333-09$03.00/0
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AGE-RELATED DECLINE IN THE BIOSYNTHESIS OF MITOCHONDRIAL INNER MEMBRANE PROTEINS

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(*Received* 18 *November* 1981)

INTRODUCTION

MITOCHONDRIA CONTAIN their own unique protein synthesizing system which produces some of the proteins of the inner mitochondrial membrane (Mason and Schatz, 1973; Tzagaloff *et al.*, 1973; Weiss, 1972; Lin *et al.*, 1974; Ibrahim and Beattie, 1976; Marcus *et al.*, 1980). In order to synthesize mitochondrial inner membrane proteins two distinct genetic systems are required, one mitochondrial and one nuclear (Schatz and Mason, 1974; Ibrahim *et al.*, 1973). Therefore, two intracellular sites of protein synthesis are involved, one mitochondrial and one cytoplasmic. The vast majority of mitochondrial inner membrane proteins are synthesized on cytoplasmic ribosomes and subsequently transferred into the mitochondria. The integration of these proteins into the mitochondrial membrane requires the presence of ten or more hydrophobic proteins synthesized within mitochondria on mitochondrial ribosomes (Ibrahim and Beattie, 1976; Marcus *et al.*, 1980; Hawley and Greenawalt, 1970). Under normal growth conditions, the assembly of mitochondrial protein proceeds in a cordinate manner via signals between the cytoplasm and mitochondria to maintain orderly synthesis (Marcus *et al.*, 1980).

Previous work from this laboratory has shown that iron deficiency results in a decrease in cytoplasmic protein synthesis via a decrease in cellular hemin (Freedman and Rosman, 1976). It also produces a decrease in isolated mitochondrial protein synthesis, but in contrast to cytoplasmic protein synthesis this effect of iron deficiency in mitochondria is independent of hemin. Indeed, hemin at concentrations of 10^{-7} M or greater is severely inhibitory to mitochondrial protein synthesis (*Marcus et al.*, 1980). In the present study we demonstrate that isolated rat liver mitochondrial protein synthesis declines with age independent of iron deficiency.

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METHODS AND PROCEDURE

Materials

Sucrose was obtained from Schwarz-Mann. Adenosine triphosphate, phosphoenolpyruvate, pyruvate kinase, cycloheximide, chloramphenicol, hemin, HEPES[1] and digitonin were obtained from the Sigma Chemical Company. The non-radioactive amino acids were from Calbiochem, and α,α-dipyridyl was from the Fisher Scientific Company. Digitonin was recrystallized in ethanol twice before use. L-(U-^{14}C) leucine, 350 Ci/mole was obtained from the Amersham Corporation and L-(3,4,5-^{3}H) leucine, 120 Ci/mmole from New England Nuclear Corporation. All other reagents were of the highest grade commercially available. All liquid scintillation counting was performed in a Nuclear Chicago Mark I spectrometer.

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Preparation of rat liver mitochondrial fractions

Young and old male Sprague-Dawley rats ranging in age from 2 weeks to 30 months were maintained on regular laboratory diets and fasted for 24 hours before each experiment. Animals were killed by decapitation and their livers were removed immediately and chilled on ice. Liver cell mitochondria were prepared under sterile conditions in a medium containing 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.6, and 0.001 M EDTA sodium salt as previously described (Marcus *et al.*, 1980).

The following precautions were taken to ensure that we were measuring isolated mitochondrial protein synthesis: (a) sterile incubations; (b) periodic sampling for bacterial culture; (c) tubes minus mitochondria were incubated and processed to show that incorporation does not occur in the absence of mitochondria; (d) there was no inhibition by cycloheximide or erythromycin but marked inhibition by chloramphenicol of the isolated mitochondria (Marcus *et al.*, 1980). In addition, blood samples were taken from the animal at the time of sacrifice to exclude iron deficiency as a cause of diminished mitochondrial protein synthesis (Marcus *et al.*, 1980).

Inner membrane-matrix fractions were prepared with digitonin as described by Schnaitman and Greenawalt (1968). The pellet obtained by centrifugation of the digitonin-treated mitochondria was washed twice by resuspending it in the same volume of sucrose-Tris-EDTA and centrifuging it at 27,000 × g for 20 minutes. The inner membrane-matrix fraction was then suspended in 3 ml of 0.01 M HEPES, 0.01 M KCl, 0.015 M magnesium acetate, 0.005 M dithiothreitol and 0.5% Triton-X 100, pH 7.6, and left on ice for 20 minutes. This fraction was then analyzed on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis as described here.

Amino acid incorporation studies

The amino acid incorporation media was similar to that previously described (Marcus *et al.*, 1980) and contained 90 mM KCl, 5mM potassium phosphate buffer, pH 7.4, 10mM $MgCl_2$, 50 mM bicine buffer, pH 7.4, 22.5 μg of a complete amino acid mixture minus leucine, 15 μm (0.5 μCi/ml) L-(U-^{14}C) leucine, 2 mM ATP, 2mM phosphoenolpyruvate, 10 μg pyruvate kinase and 2-3 mg mitochondrial protein in a final volume of 2 ml. The incubations were for 30 minutes at 37° in a shaking waterbath with air as the gas phase. Incubations were terminated by addition of 10 mM non-radioactive L-leucine followed by precipitation of protein with 5% trichloroacetic acid.

The labeled proteins were prepared for liquid scintillation counting by previously described methods (Marcus *et al.*, 1980). Protein concentration was determined by the method of Lowry *et al.* (1951). The preparation and addition of different agents to the incubation media are as described in the tables and figure.

Identification of mitochondrial inner membrane-matrix proteins

In these experiments, the incubation conditions were identical to those described above except that L-(3,4,5-^{3}H) leucine was substituted for L-(U-^{14}C) leucine. The concentration of L-(3,4,5-^{3}H) leucine was 15 μM and 10 μCi/ml were added. After 30 minutes incubation the inner membrane-matrix fraction was prepared as described above and 100 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels. Electrophoresis was performed by the method of Weber and Osborn (1969) at room temperature on 9 cm tube gels at a constant current of 3.0 mA/gel for 16 hours.

Protein bands were visualized by staining with Coomassie Brilliant Blue and gels scanned at 570 nm in a Gilford model 240 recording spectrophotometer equipped with a linear transporter. Identical gels were sliced into 1.6 mm sections, dissolved by heating with 0.5 ml of 30% H_2O_2 for 2 hours, and counted in "Aquasol". Recovery of radioactivity from the gels was 95%.

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[1]N-2-hydroxyethylpiperazine N^{1}-2 ethane sulfonic acid.

TABLE 1. EFFECT OF AGE ON INCORPORATION OF L-(U-^{14}C) LEUCINE INTO PROTEIN OF ISOLATED RAT LIVER MITOCHONDRIA INCUBATED IN VITRO (n = 10)[a]

Young (1–3 months)	Old (26–30 months)
cpm/mg mitochondrial protein ± SEM	cpm/mg mitochondrial protein ± SEM
8,468 ± 254	4,741 ± 315[b]

[a] Incubations were as described in Methods & Procedure for 30 minutes at 37°C in triplicate with less than 10% variation between samples.

[b] Old statistically differs from young by student t test analysis ($p < 0.001$).

RESULTS

Effect of age on rat liver mitochondrial protein synthesis

The effect of age on isolated rat liver mitochondrial protein synthesis is shown in Table 1. There was a mean 46% decrease in mitochondrial protein synthesis in those organelles isolated from old animals (26–30 months) as compared to those from young rats (1–3 months). The mean serum iron/total iron binding capacities in these animals were 221/346 μg per 100 ml for the old animals, and 253/447 μg per 100 ml for the young animals, corresponding to ratios of 0.64 and 0.53 respectively, which are both well within the normal ranges. None of the animals used was iron deficient. These results exclude iron deficiency as a cause of the diminished mitochondrial protein synthesis.

Decline in mitochondrial protein synthesis with age

Fig. 1 shows the gradual decline in isolated mitochondrial protein synthesis with age. Rats are sexually mature at approximately 1-month-of-age and within 1 to 2 months after sexual maturity show a decline in the ability of their mitochondria to synthesize protein. Throughout the life span of these animals this becomes more marked, and rats over 2-years-of-age only synthesize at approximately 40% of the rate of 2-8-week-old animals. A representative rate of incorporation is shown in Fig. 2. The "young" animal used in this experiment was 8 weeks old, while the "old" animal was 112 weeks old. As can be seen the initial rate of incorporation was decreased in the "old" animal and was 145 cpm/mg/minute compared to 320 cpm/mg/minute in the "young" animal.

Effect of age on mitochondrial inner membrane biosynthesis

In order to further demonstrate that the effect of age was on mitochondrial inner membranes, this subcellular component was isolated as described in Methods and Procedure. In Fig. 3 (upper panel) a representative labeling pattern on SDS-polyacrylamide gel electrophoresis from a 30-minute incubation of liver mitochondria from a young animal (6 weeks) is shown. In the middle panel the labeling pattern from an old (118 weeks) animal is shown. As can be seen, there is a generalized decrease in labeling which appears to be most marked in the high molecular weight fractions from the aged animal. The protein staining pattern with Coomassie Blue (lower panel) was identical for mitochondria from

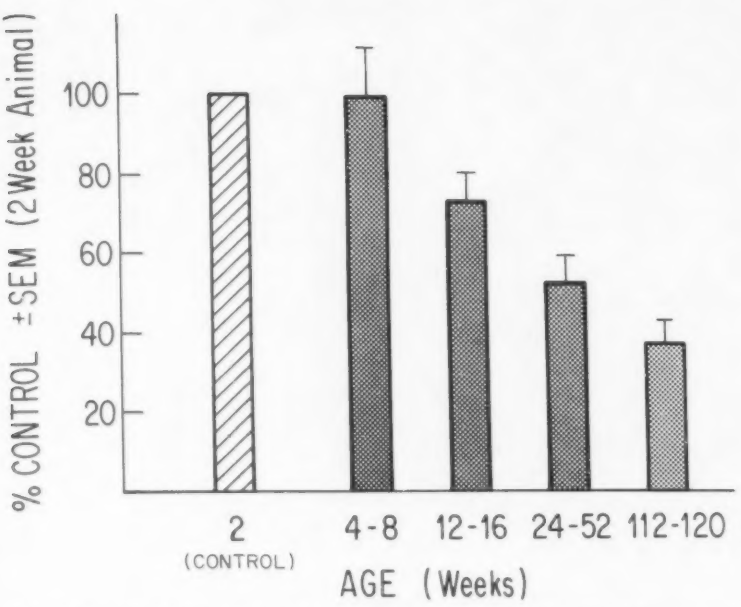

FIG. 1. Effect of Age on Isolated Mitochondrial Protein Synthesis from Rat Livers. Incubations in triplicate were for 30 minutes at 37°C as described in Experimental Procedures. Each age shown is the mean ± SEM of 6 separate experiments performed in triplicate with less than 10% variation between duplicates. Each value was corrected by subtracting a small zero time. The mean specific activity of the 2-week-old animals was 8,238 cpm/mg mitochondrial protein.

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both young and old animals. In these experiments 100 μg/ml cycloheximide did not alter the labeling pattern while 100 μg/ml chloramphenicol almost totally inhibited incorporation, confirming that we were indeed measuring the mitochondrial membrane proteins (Beattie *et al.*, 1967).

Effect of stimulators and inhibitors of mitochondrial protein synthesis in aged animals

The ability of mitochondria from aged animals to respond to stimulation or inhibition by agents previously reported to cause stimulation of mitochondrial protein synthesis i.e., ferrous ammonium sulphate (5×10^{-4}M) and hemin (10^{-8}M), as well as an inhibitor, α,α-dipyridyl (2×10^{-4}M) (Marcus *et al.*, 1980) is shown in Table 2. The results of experiments from 6 young and 3 old pooled rat livers are reported. Ferrous ammonium sulphate stimulated mitochondrial protein synthesis 1.62 fold in the young animals' mitochondria but not in the old animals'. Similarly, hemin stimulated the young animals' mitochondria 1.34 fold, but not the old rats. It appeared that the effect of ferrous ammonium sulphate and hemin have been somewhat inhibitory in the old animals. When the ferrous iron chelator, α,α-dipyridyl, was added to the isolated mitochondria it inhibited the young rats' mitochondria by 38% and the old rats' mitochondria by 44%.

Experiments were repeated using L-(^{35}S) methionine in order to be certain that the difference in incorporation was not due to a defect in leucine transport into the mitochon-

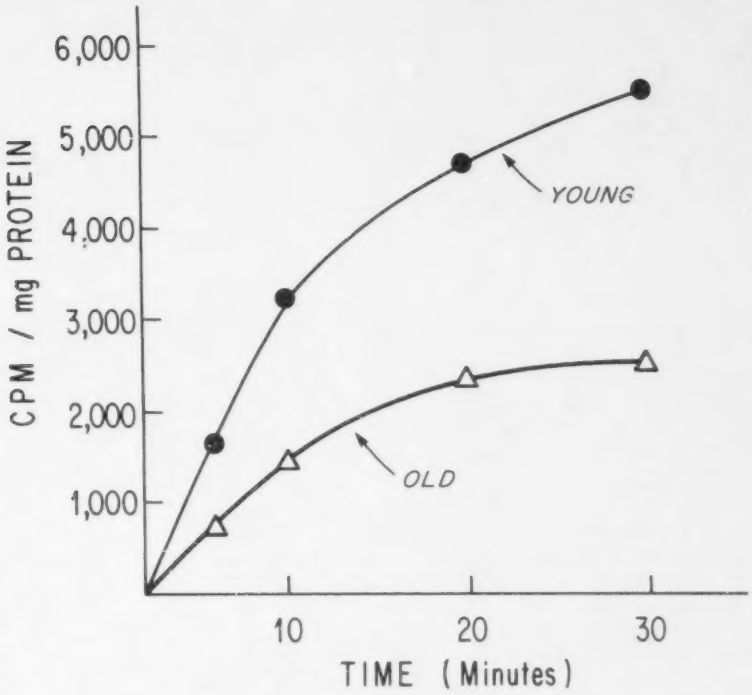

FIG. 2. Rate of Incorporation of L-(U-^{14}C) Leucine into Protein of Isolated Mitochondria of Young and Old Rat Livers. Incubations at 37°C were as described in Experimental Procedures. Samples were withdrawn at the time shown. This is a representative experiment performed in triplicate with less than 10% variation between duplicates. A small zero time was subtracted from each value. The young animal was 8 weeks old and the old animal was 112 weeks.

dria. Mitochondria from 28-month old rats incorporated 37% less methionine than did mitochondria from 2-month old rats.

Mitochondrial amino acid pools

The size of the leucine pool in mitochondria from young and old rats was also measured. Mitochondria were incubated with L-(^{14}C) leucine for 30 minutes as described in Methods and Procedure, washed with isolation media (5 volumes) until the washings were free of counts, lysed by the addition of 2 ml 5% TCA, and the amount of radiolabel in the supernatants and quantity of mitochondrial protein determined. There were 876 cpm/mg and 784 cpm/mg released by the old and young mitochondria respectively. Under the conditions of this assay the old rat mitochondria incorporated 5341 cpm/mg as compared to 8772 cpm/mg in the young. Mitochondria from young and old rats were incubated with L-(^{14}C) leucine for 30 minutes as described in Methods and Procedure, washed with isolation medium, and reincubated with non-radioactive leucine for an additional 30 minutes. No significant leakage of label into the medium from either the old or the young mitochondria was found.

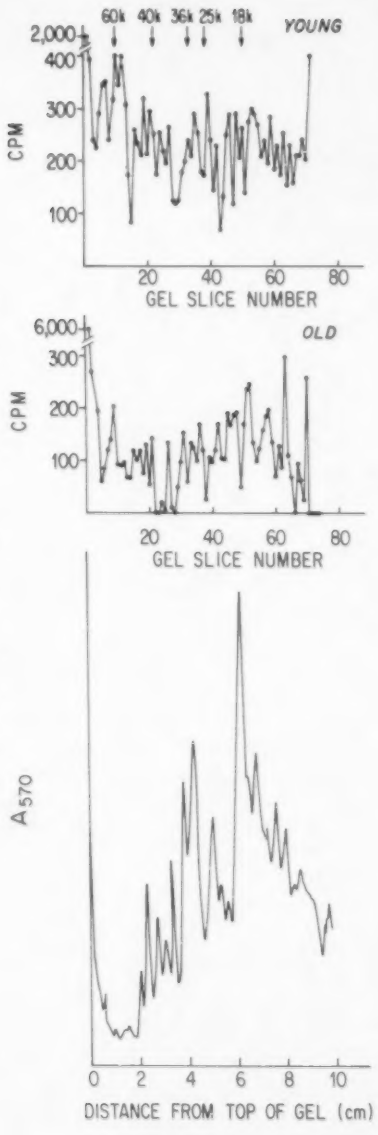

FIG. 3. Effect of Age on L-(3,4,5-^{3}H) Leucine Incorporation into rat liver Mitochondrial Inner Membrane-Matrix Proteins. The incubations, preparation of inner membrane-matrix fraction with digitonin and polyacrylamide gel electrophoresis were carried out as described in Experimental Procedures. Upper tracing-young animal (age 6 weeks); middle tracing-old animal (age 118 weeks); bottom tracing-A_{570} scan of upper and middle pattern. The molecular weight of the various peaks was estimated from a plot of the M_r *vs.* migration distance employing the following markers of known molecular weight: bovine serum albumin (68,000); aldolase (40,000); chymotrypsinogen (25,700); ribonuclease (14,000). Each sample containing 100 μg of protein and 20,000 cpm (young) and 15,000 cpm (old), 1% SDS, 1% β-mercaptoethanol was heated for 2 minutes at 100°. The samples were then electrophoresed on 10% polyacrylamide gels.

DISCUSSION

Previous work from this laboratory has shown that iron is a requirement for maximal mitochondrial protein synthesis independent of hemin (Marcus *et al.*, 1980). The present study shows that mitochondrial protein synthesis gradually declines with advancing age of the animal. Rats, 112-210-weeks-of-age, synthesize mitochondrial proteins at only 40% of the rate of 4-8-week-old rats. The mean serum iron/total binding capacity for the aged animals was close to that of the young ones, indicating that the decline in mitochondrial protein synthesis with advancing age is not due to iron deficiency. The response of the

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TABLE 2. EFFECT OF FERROUS AMMONIUM SULPHATE, HEMIN AND α,α-DIPYRIDYL ON ISOLATED LIVER MITOCHONDRIAL PROTEIN SYNTHESIS FROM YOUNG & OLD RATS[a]

Source of mitochondria	Addition	% young control[b]
Young	None	100
	Ferrous ammonium sulphate (5×10^{-4}M)	162
	Hemin (10^{-8}M)	134
	α,α-Dipyridyl (2×10^{-4}M)	62
Old	None	70
	Ferrous ammonium sulphate (5×10^{-4}M)	57
	Hemin (10^{-8}M)	52
	α,α-Dipyridyl (2×10^{-4}M)	36

[a] Incubations were as described in Methods & Procedure for 30 minutes at 37°C in triplicate with less than 10% variation between samples.

[b] The specific activity of the young control was 9,970 cpm/mg mitochondrial protein.

mitochondria from aged animals to α,α-dipyridyl further confirms that mitochondrial iron deficiency was not the cause of this decrease in mitochondrial protein synthesis. In addition, the decrease in mitochondrial protein synthesis in the aged animal is due to neither a smaller amino acid pool size nor an increase in leakage of radiolabeled amino acid from the mitochondria of the aged rat.

Protein synthesis in mitochondria from old rats may be inhibited by the ferrous iron chelator, α,α-dipyridyl. However, stimulation by either 5×10^{-4}M ferrous ammonium sulphate or 10^{-8}M hemin did not occur, indicating that not only are mitochondria from old rats unable to synthesize inner mitochondrial membrane proteins at the same rate as young rats, but also cannot be stimulated to do so.

The results reported here suggest that in old rats the synthesis of proteins on mitochondrial ribosomes decreases coordinately with the previously reported decline in cytoplasmic protein synthesis (Moldave *et al.*, 1979). It is known that when cytoplasmic protein synthesis is primarily inhibited with cycloheximide *in vivo*, mitochondrial protein synthesis secondarily diminishes (Ibrahim and Beattie, 1976). We have previously postulated (Marcus *et al.*, 1980) that this could occur through cytoplasmic proteins (e.g., the hemin-controlled repressor (Freedman and Rosman, 1976; Freedman *et al.*, 1974) or iron or hemin concentrations. However, it is not clear whether the protein synthetic decrease seen with age is primarily in the mitochondria or the cytoplasm or if it occurs in both compartments independently, and it remains to be shown if and how the cytoplasm and mitochondria communicate to coordinate protein synthesis.

Paterniti and Beattie (1979) have reported a molecular weight of 58,000 for δ-aminolevulinic acid synthase on SDS polyacrylamide gels, purified from uninduced rat liver mitochondria. Our finding of a decrease in incorporation of label into the 60,000

molecular weight range seen on SDS polyacrylamide gel electrophoresis of the mitochondrial inner membranes isolated from aged rats might explain the decrease in δ-aminolevulinic acid synthase activity reported in elderly rats (Paterniti *et al.*, 1978). This rate-limiting enzyme of heme synthesis (Levere and Granick, 1967) is synthesized in the cytoplasm under nuclear DNA control (Hayashi *et al.*, 1969; Beattie and Stuchell, 1970), transported into the mitochondria and bound to a protein on the inner mitochondrial membrane (Hayashi *et al.*, 1969; Beattie and Stuchell, 1970; Rosenberg and Marcus, 1974) which is synthesized on mitochondrial ribosomes under the control of mitochondrial DNA. Maximal cellular activity of this enzyme results from this protein-protein interaction. Conceivably, the decrease in cellular δ-aminolevulinic acid synthase reported previously (Paterniti *et al.*, 1978) is ultimately due to the decrease in mitochondrial protein synthesis seen with age, which we report here.

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SUMMARY

Isolated mitochondria from rat livers of various ages show a gradual decline in the rate of inner membrane-matrix protein synthesis with advancing age of the animal. Rats at 112-120 weeks synthesize these proteins at only 40% of the rate of 2-8-week-old animals. The initial rates of incorporation of label were 145 cpm/mg/minute for the "old" animals, and 320 cpm/mg/minute for the "young" animal. No difference in either amino acid pool size or leakage of label through the mitochondrial membrane was detected in the two age groups. Treatment of the mitochondria with ferrous ammonium sulphate produced a 1.62 fold increase in mitochondrial protein synthesis in the young animal but not in the old. Hemin treatment produced a similar effect.

These results suggest that the decrease in δ-aminolevulinic acid synthase activity seen with age (Paterniti *et al.*, 1978) may be due to a decrease in the synthesis of mitochondrial inner membrane proteins.

Acknowledgements—This research was supported by PHS Grants AG 01399 from the National Institute of Aging, and AM 13532 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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Experimental Gerontology, Vol. 17, pp. 343–357, 1982
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0531-5565/82/050343-15$03.00/0
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INFLUENCE OF DRUGS ON MACROMOLECULAR SYNTHESIS DURING CELL SYNCHRONIZATION

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(*Received* 15 *December* 1981)

Abstract—A cell culture system has been used to examine the effect of various pharmacologic agents on DNA synthesis with the hope of utilizing this system for the evaluation of drugs at the cellular level. Glucocorticoids have been shown to have a differential effect on growth dependent on the cell type studied. For this reason steroidal anti-inflammatory agents were chosen to study in our culture system. Aspirin, a non-steroidal anti-inflammatory agent, was also studied for a comparison with glucocorticoids. The CNS stimulants, caffeine and amphetamines, were studied for their effects on non-target cells in culture and compared with the response of target cells. Doxorubicin, an anti-neoplastic agent, has been shown to depress growth in a variety of cells. This drug was also studied in our culture system. We have found that steroidal anti-inflammatory drugs induced a dose-dependent stimulation of DNA synthesis in normal human fibroblasts that was also age-dependent, while decreasing DNA synthesis in SV40 virus transformed cells. Aspirin (25 μg/ml) exhibited a similar response. Human fibroblasts were found to be responsive to the CNS stimulants, exhibiting a dose-dependent decrease in DNA synthesis when exposed to caffeine. Amphetamine (200 μM) depressed DNA synthesis in normal fibroblasts and increased it in SV40 virus transformed cells. All cells studied exhibited a depression of DNA synthesis when treated with doxorubicin (0.1 μg/ml).

INTRODUCTION

CELL CULTURE provides a convenient means for studying various cell types at macromolecular and cellular levels in a controlled environment. It is well established that human diploid fibroblasts have a finite life span *in vitro* undergoing an initial period of rapid proliferation followed by a gradual decline in the growth rate and ability to initiate DNA synthesis (Hayflick, 1965). Heteroploid cultures, such as SV40 virus transformed cells on the other hand, can be grown in culture indefinitely. This allows us to study the parameters of aging and transformation and the effects of addition of various drugs to the medium in cells in culture as has been studied previously (Grove and Cristofalo, 1977; Kaji *et al.*, 1980 Lamon *et al.*, 1982).

Steroidal anti-inflammatory agents, such as hydrocortisone or prednisolone, have been shown to increase the proliferating capacity of WI-38 human fetal lung fibroblasts in

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culture (Grove and Cristofalo, 1977; Rosner and Cristofalo, 1974) while the growth of cells transformed with SV40 virus is inhibited (Young and Dean, 1980). The effect appears to be cell, species and steroid specific. Non-steroidal anti-inflammatory agents, such as aspirin, inhibit cell growth in both normal and tumor cells (Hial *et al.*, 1976; 1977; Bayer and Beaver 1979; Bayer *et al.*, 1979, 1980).

CNS stimulants such as caffeine and amphetamines have also been studied for their effects on cell growth. Caffeine, a methylated xanthine, has been shown to produce chromosomal aberrations and cell death through alkylation of DNA strands (Kihlman, 1977). It has been shown to inhibit DNA synthesis in normal cells but not in polyoma virus transformed cells (O'Neill, 1979). Amphetamines, a catecholamine-like drug, have been shown to produce a dose dependent inhibition of protein synthesis (Widelitz *et al.*, 1975; Widelitz and Hable, 1980; Salomon, 1978). The anti-neoplastic agent, doxorubicin, appears to inhibit cell growth not only by intercalation into DNA strands but also by inhibiting directly DNA polymerases (Tanaka and Yoshida, 1980).

All of the above drugs have been shown to affect cell growth in some way. The studies presented here were undertaken to examine the effect of these pharmacologic agents on DNA synthesis in various cells in culture in a hope to utilize this culture system for the evaluation of drugs at cellular level. Normal human fibroblasts, early and late passages, and their SV40 virus transformed counterparts were used with all drugs studied. In addition, mouse 3T3 fibroblasts and its SV40 transformed counterpart, mouse brain glial cells and neuroblastoma cells were used in some of the studies.

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MATERIALS AND METHODS

Cell Culture

Human lung fibroblast strains IMR-90; AG 2804, an SV40 virus transformant of IMR-90 (SV-IMR90); WI-38; and VA132RA, an SV40 virus transformant of WI-38 (SV-WI38); mouse 3T3 fibroblasts and its SV40 virus transformed counterpart (SV-3T3) as well as mouse brain cells (MBC) and neuroblastoma cells (NA) were used in these studies. All cultures were maintained in minimum essential medium (MEM) supplemented with 5% fetal calf serum. The cells were grown at 36°C in a humidified, 5% CO_2 atmosphere in a Linbro 24-well dish.

Before cells approached confluency, each well was washed twice with MEM without fetal calf serum and then incubated with MEM free of fetal calf serum. Unless otherwise noted, after 48–60 hours incubation in the absence of fetal calf serum, the MEM was removed and the cells were washed once with MEM. The cells were fed with one ml MEM containing fetal calf serum to initiate the cell cycle. At this time the appropriate drug was added to test cultures.

Experimental procedure

To test ^{3}H-thymidine incorporation and DNA synthesis at various hours after the cell cycle was initiated, wells were washed twice with MEM and 0.5 ml of MEM containing ^{3}H-thymidine was added. The plate was further incubated for one hour. After incubation the cells were washed three times with phosphate buffered saline–PBS (7.650 *g* NaCl/0.725 *g* Na_2HPO_4/0.212 *g* KH_2PO_4 in 1 liter) and dissolved in 0.2 ml of 0.3% sodium dodecylsulfate. For measurement of ^{3}H-thymidine uptake, an aliquot of 0.05 ml was processed for trichloroacetic acid (TCA) insoluble radioactive measurement (Mans and Novelli, 1961) and counted in a liquid scintillation counter (Beckman). An aliquot of 0.10 ml was used to determine protein concentration by the method of Lowry (1951).

Drugs

The drugs studied were hydrocortisone (Upjohn), prednisolone (Merck, Sharp and Dohme), aspirin (Fisher Scientific), d-amphetamine sulfate (SKF), caffeine (Aldrich Chemical), doxorubicin (Adria Laboratories), and dexamethasone (Organon).

RESULTS

In order to study the effects of agents on DNA synthesis of various cell types, populations of synchronized cells were prepared. In these studies synchronization was accomplished through deprivation of serum. Cultures of IMR-90 fibroblasts were fasted for 36 to 72 hours and their progress through the cell cycle after feeding was followed through incorporation of ^{3}H-thymidine.

Acceptable synchronization of young normal fibroblasts (Population Doubling Level—PDL 26) is obtained with starvation times ranging from 36–60 (Fig. 1). Lengthening the starvation period results in broader *S* phases and a less noticeable shift from the first cycle to the second. This suggests that DNA synthesis may be regulated directly by the modes of starvation. Older normal fibroblasts (PDL 50) (Fig. 2) require longer starvation periods (ranging from 48–72 hours) for the entire cell population to enter an acceptable new synchronization cycle. Thirty-six hours is insufficient for synchronization for older cells due to the longer generation time of senescent cells.

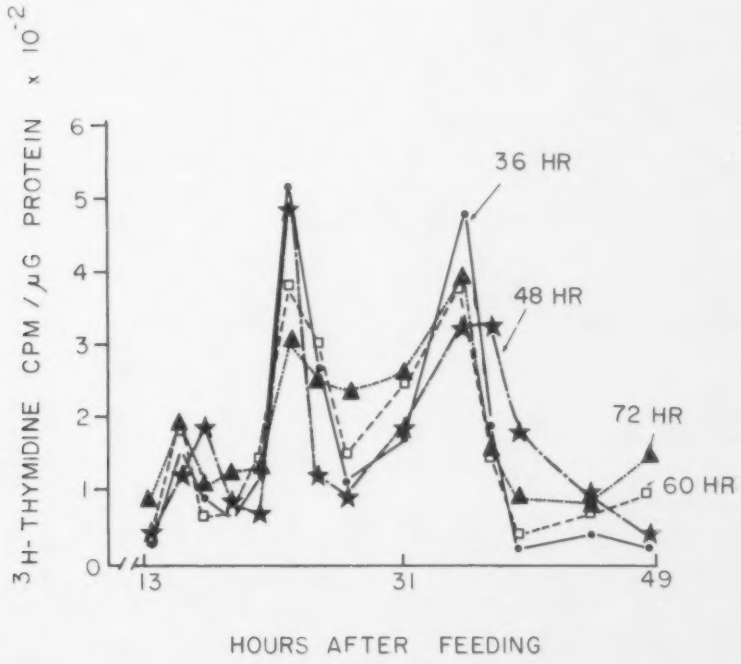

FIG. 1. Effect of starvation periods on synchronization of young fibroblasts (PDL 26). Cultures of IMR-90 Fibroblasts, PDL 26, were fasted for 36, 48, 60 and 72 hrs. After the addition of fetal calf serum, samples were pulsed for 1 hr with 0.5 ml of MEM containing 1.0 μCi/ml ^{3}H-thymidine at various incubation intervals. ^{3}H-Thymidine uptake was measured as radioactivity precipitable by cold trichloroacetic acid (Mans and Novelli, 1961). An aliquot of each sample was used to determine protein concentration.

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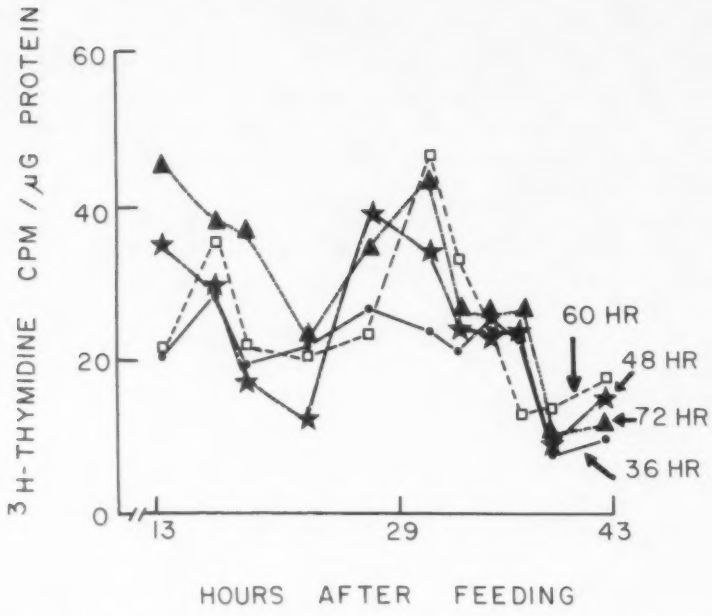

FIG. 2. Effect of starvation periods on synchronization of senescent fibroblasts (PDL 50). Cultures of IMR-90 fibroblasts, PDL 50, were fasted and processed as in Fig. 1.

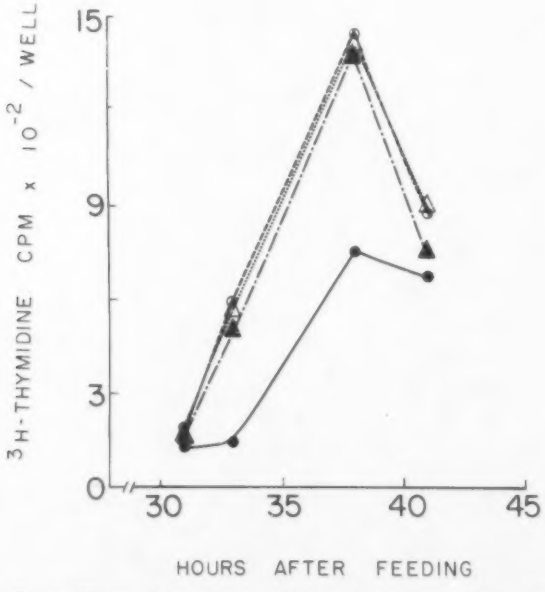

FIG. 3. Effects of steroids on DNA synthesis. Young (PDL 35) IMR 90 fibroblasts were synchronized with a fasting period of 36 hrs. They were then fed with MEM containing fetal calf serum alone (●) or with 10.0 µg/ml dexamethasone (0), 10.0 µg/ml prednisolone (▲), 10.0 µg/ml hydrocortisone (△).

Anti-inflammatory drugs

Studies on the ^{3}H-thymidine incorporation into acid precipitable material by synchronized cells in the presence of the anti-inflammatory agents such as dexamethasone, hydrocortisone, prednisolone, and aspirin were undertaken. Fig. 3 shows the results for the effect of steroidal drugs on young (PDL 35) IMR-90 fibroblasts. All three steroids were equally potent in stimulation of these cells into the *S* phase, and the stimulation above the control continued into the succeeding cell cycle. A dose of 10 μg/ml of the steroidal drugs stimulated the incorporation of thymidine into the cold trichloroacetic acid (TCA) insoluble fraction by more than 50%. When different concentrations of prednisolone in the range of 0.1-10.0 μg/ml were tested, even the dose as low as 0.1 μg/ml showed the stimulatory response (Fig. 4) indicating higher potency of the drug as reported (Haynes and Larner, 1974). These steroidal agents facilitated entry into the *S* phase of the cell cycle in late passage cells (PDL 50) but did not increase total DNA synthesis. This steroid stimulation on DNA synthesis can only be seen when the cells are primed for DNA synthesis by fetal calf serum. In the absence of serum no stimulatory effect of these agents was seen (Fig. 5).

It has been reported that the protein content per cell is constant for a given cell type and in IMR90 and WI38 fibroblasts, within a PDL (population doubling level) (Hara *et al.*, 1981; Kaji *et al.*, 1980). Therefore, we have chosen to express our results as cpm per μg protein.

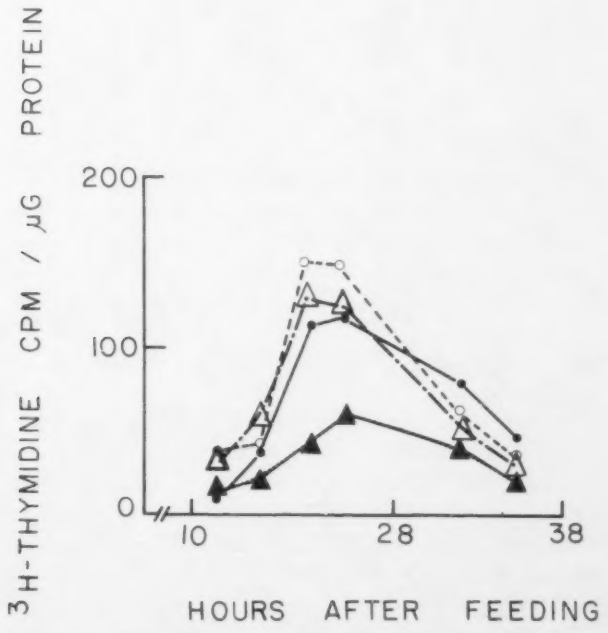

FIG. 4. Dose response of glucorticoid drugs on young IMR 90 fibroblasts (PDL 26). Cultures were fasted for 36 hrs and then fed with MEM containing fetal calf serum alone (▲) or with 0.1 μg/ml prednisolone (●), 1.0 μg/ml prednisolone (○), 1.0 μg/ml dexamethasone (△).

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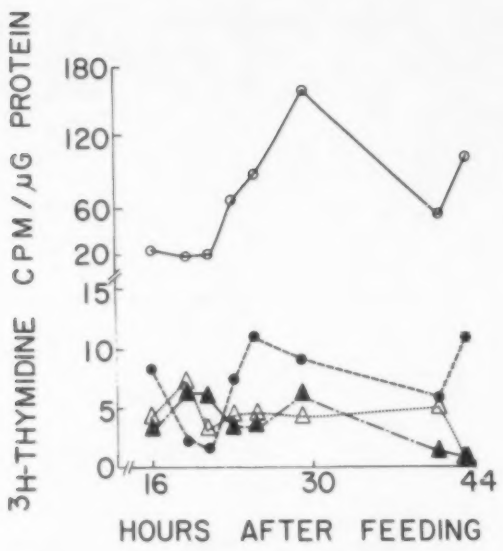

FIG. 5. Effect of steroids on DNA synthesis in the absence of fetal calf serum. Young (PDL 35) IMR90 fibroblasts were synchronized with a fasting period of 45 hrs. They were then fed with MEM alone (●) or containing: 5% fetal calf serum (○), 10 μg/ml dexamethasone (□), 10 μg/ml hydrocortisone (▲). Samples were taken at various intervals of the cell cycle and pulsed for 1 hr. with MEM containing 1.0 μCi/ml ^{3}H-thymidine. Aliquots were then processed for cold trichloroacetic acid precipitable radioactivity and protein concentrations were determined.

Table 1 shows the incorporation of ^{3}H-thymidine into DNA in other cell types tested. The 3T3 fibroblasts showed a significant increase in DNA synthesis when treated with 10 μg/ml of any of the steroids studied. The SV3T3 fibroblasts on the other hand showed a dramatic decrease when exposed to the same dosage of these drugs. This is consistent with previous reports (Young and Dean, 1980). MBC and NA cells also showed a decreased incorporation of nucleic acid into DNA. Both SV-IMR90 (AG2804) and SV-W138 (VA132RA) showed no significant change in DNA synthesis when compared with untreated cells.

Fig. 6 shows the response of IMR-90 fibroblasts, both early and late passages, to the presence of a non-steroidal anti-inflammatory agent, aspirin (25 μg/ml), in the incubating media. There is a stimulation of total DNA synthesis in young fibroblasts at this dosage and this increase (50% above untreated cells) continues to be seen in the succeeding cell cycle. As can be seen from Fig. 6 and Table 2, older IMR90 fibroblasts show no significant change in nucleic acid incorporation. The SV-IMR90 cells exhibit a significant reduction in DNA synthesis when exposed to 10 μg aspirin/ml, but no significant changes can be observed when the concentration is increased. Other investigators have reported an inhibition of cell growth by nonsteroidal anti-inflammatory agents (indomethacin and aspirin) with a decrease in protein and DNA synthesis as well as cell number (Bayer and Beaven, 1979; Bayer *et al.*, 1979, 1980; Hial *et al.*, 1976, 1977; Lynch, *et al.*, 1978). This inhibi-

TABLE 1. EFFECT OF STEROIDAL ANTI-INFLAMMATORY AGENTS ON ^{3}H-THYMIDINE INCORPORATION

	Prednisolone 10 μg/ml	Dexamethasone 10 μg/ml	Hydrocortisone 10 μg/ml
	% Control		
3T3	182.2 ± 0.6 (2)[c]	177.0 ± 2.2 (2)[c]	166.0 ± 2.0 (2)[c]
SV3T3	63.2 ± 13.2 (4)[b]	47.3 ± 1.1 (2)[c]	25.3 ± 1.9 (2)[c]
MBC	42.7 ± 3.0 (4)[c]	31.8 ± 0.8 (2)[c]	40.6 ± 0.8 (2)[c]
NA	65.7 ± 16.5 (4)[a]	81.8 ± 2.3 (2)[a]	67.3 ± 1.1 (2)[c]
SV-IMR90 (AG2804)	102.7 ± 4.1 (3)		
SV-WI38 (VA132RA)	106.1 ± 12.9 (3)		

[a] $p < 0.02$
[b] $p < 0.01$
[c] $p < 0.001$

Experimental conditions are described in Methods. The sum of radioactivity per μg protein incorporated into cold TCA precipitable fraction from four (4) points defining peak DNA synthesis (*S* phase) for synchronized cultures was used. Values presented are mean ± S.D. and are percentages of control cultures. Number in parenthesis represents number of sample cultures.

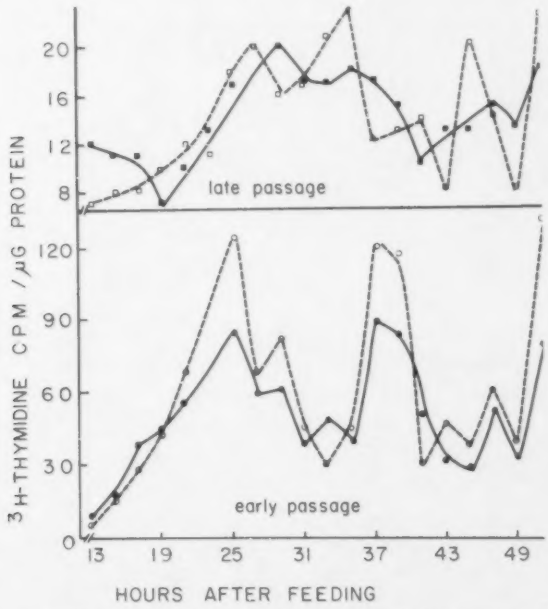

FIG. 6. Effects of aspirin on ^{3}H-thymidine uptake by young and old cells. Synchronized cultures of IMR-90 young and old were fed with MEM containing fetal calf serum with or without 25 μg/ml aspirin. Samples were taken at various times over a 52-hr. period after the addition of serum. Each sample was pulsed with MEM containing 1.0 μCi/ml ^{3}H-thymidine for 1 hr. Aliquots were processed for determination of cold trichloroacetic acid precipitable radioactivity and protein. IMR-90 young cells: control (●), 25 μg/ml aspirin (○). IMR-90 old cells: control (■), 25 μg/ml aspirin (□).

TABLE 2. EFFECT OF ASPIRIN ON ^{3}H-THYMIDINE INCORPORATION

	Doses		
	10 μg/ml	25 μg/ml	50 μg/ml
	% Control		
IMR90 PDL 33	123.7 ± 28.5 (3)	150.0 ± 12.2 (3)[a]	194.5 ± 79.1 (3)
PDL 50	164.4 ± 50.4 (3)	97.7 ± 6.0 (4)	94.3 ± 17.9 (3)
SV-IMR90	43.9 ± 34.4 (3)[b]	97.8 ± 13.9 (4)	116.5 ± 13.8 (3)

[a] $p < 0.01$
[b] $p < 0.05$

Experimental conditions are described in Methods. The sum of radioactivity per μg protein incorporated into cold TCA precipitable fraction from four (4) points defining peak DNA synthesis (*S* phase) for synchronized cultures was used. Values presented are mean S.D. and are percentages of control cultures. Number in parenthesis represents number of sample cultures. PDL is population doubling level and is indicative of the relative age of the fibroblasts.

tion was seen in both hepatoma cells and human foreskin fibroblasts with dosages 15–30 times higher than those used in our studies. Hial and co-workers (1977) reported an increase in nucleotide incorporation using low concentrations of aspirin (2 mM), a dose approximately 10 times higher than used in our studies and a dose-dependent inhibition with higher concentrations.

TABLE 3. EFFECT OF AMPHETAMINE ON ^{3}H-THYMIDINE INCORPORATION

	Doses	
	100 μM	200 μM
	% Control	
IMR90 PDL 33	84.5 ± 14.6	79.2 ± 12.8[c]
PDL 50	95.5 ± 12.2	88.3 ± 1.7[a]
SV-IMR90	91.8 ± 16.2	162.2 ± 4.7[a]
W138 PDL 33		52.3 ± 2.2[b]
PDL 50		118.4 ± 40.7
SV-WI38		144.3 ± 14.6[b]

[a] $p < 0.001$
[b] $p < 0.01$
[c] $p < 0.05$

Experimental conditions are described in Methods. The sum of radioactivity per μg protein incorporated into cold TCA precipitable fraction from four points defining peak DNA synthesis (*S* phase) for synchronized cultures was used. Values presented are mean ± S.D. and are percentages of control cultures. Each mean represents triplicate experiments except for W138, PDL 33 and 50, where duplicate experiments are reported.

PDL is population doubling level and is indicative of the relative age of the fibroblasts.

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CNS stimulants

The effect of amphetamine on the cell cycle was studied using synchronized cell cultures. Both IMR90 and WI38 young fibroblasts showed a significant decrease in DNA synthesis when exposed to 200 μM amphetamine (Table 3). This effect was age-dependent, thus late passage WI38 fibroblasts showed no significant changes. The SV40 virus transformed counterparts of these fibroblasts showed a significantly increased incorporation of nucleic acid into DNA when exposed to the same concentration. This increase was also seen with SV-IMR90 fibroblasts. No significant changes were observed with lower dosages.

Synchronized cell cultures were exposed to caffeine (0.1 mM-5.0 mM) and the incorporation of ^{3}H-thymidine into cold TCA precipitable material was measured. Table 4 shows the results of such studies for the various cells. Young human fibroblasts, both IMR90 and WI38 show a marked reduction in DNA synthesis (48.9% and 42.6%, respectively) when exposed to 1.0 mM caffeine. Older fibroblasts of these two cell strains appear to be less sensitive to this inhibition showing no significant alterations in thymidine incorporation into DNA. The SV-IMR90 fibroblasts also show an inhibition of DNA synthesis upon incubation with caffeine. 3T3 fibroblasts exhibited a significant decrease (26.7%) in total DNA synthesis when incubated with 0.1 mM caffeine. Although SV3T3 cells showed no statistically significant changes in thymidine incorporation at 0.1 mM, there appeared to be a slight increase at 1.0 mM (Table 4). Tolmach and co-workers (1977) also observed

TABLE 4. EFFECT OF CAFFEINE ON INCORPORATION OF ^{3}H-THYMIDINE

	Doses		
	0.1 mM	0.5 mM	1.0 mM
	% Control		
IMR90 PDL 33	95.8 ± 24.8	71.4 ± 16.1[c]	51.1 ± 5.9[a]
PDL 50	87.6 ± 1.2[a]	84.0 ± 25.5	113.0 ± 42.7
SV-IMR90	63.2 ± 28.5[b]	54.3 ± 41.0	67.9 ± 27.0
WI38 PDL 33	92.5 ± 23.3	76.4 ± 23.7	57.4 ± 11.4[c]
PDL 50	102.8 ± 12.2	95.4 ± 10.0	150.0 ± 70.7
MBC	100.0 ± 18.9		96.1 ± 23.3
NA	87.4 ± 11.2		74.6 ± 9.3
3T3	73.3 ± 3.5[c]		101.8 ± 18.8
SV3T3	99.5 ± 8.6		104.2 ± 0.07[a]

[a] $p < 0.001$
[b] $p < 0.01$
[c] $p < 0.02$

Experimental conditions are described in Methods. The sum of radioactivity per μg protein incorporated into cold TCA precipitable fraction from four points defining peak DNA synthesis (*S* phase) for synchronized cultures was used. Values presented are mean ± S.D. and are percentages of control cultures. Each mean represents quadruplicate experiments for IMR90 and SV-IMR90 and duplicate experiments for all other cells studied.

PDL is population doubling level and is indicative of the relative age of the fibroblasts.

an increased incorporation of thymidine into DNA in HeLa S3 cells exposed to 1.0 mM caffeine. It was of large magnitude, highly labile and appeared to be unpredictable.

It has been reported that exposure of synchronized HeLa S3 cells to 1.0 mM caffeine during the *G1* phase retards the entrance of these cells into the *S* phase of the cell cycle by 1.5 hours (Tolmach *et al.*, 1977). This retardation is even greater at 2 mM. We see this delayed initiation of DNA synthesis at a dose as little as 0.1 mM in young IMR-90 cells and to an even greater extent at 5.0 mM caffeine (Fig. 7). The extent of the delay seems to be dose dependent as is the reduction in DNA synthesis.

Cultures of MBC and NA showed no significant changes in thymidine incorporation into the acid precipitable fraction when incubated with caffeine (0.1-1.0 mM). Delayed entry into the *S* phase is seen; however, in MBC cultures exposed to 0.1 mM (Fig. 8). This shift in the cell cycle was not seen at higher doses nor in the NA cultures.

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Anti-neoplastic agents

Doxorubicin (0.1 μg/ml) was incubated with various synchronized cell cultures. IMR-90

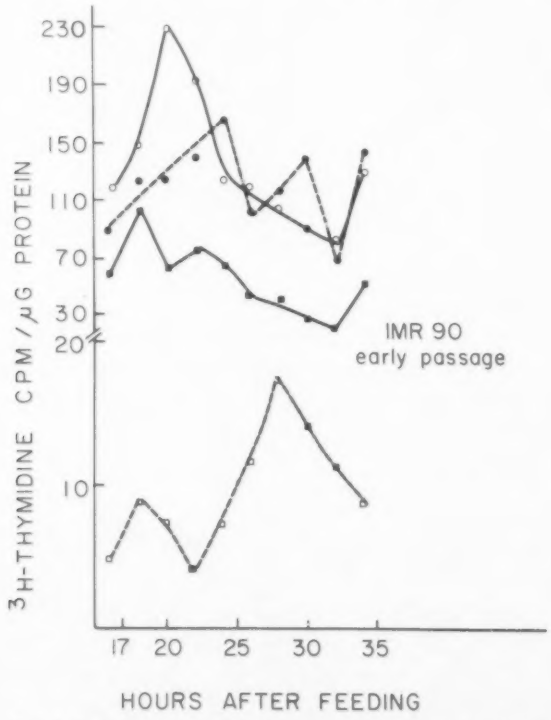

FIG. 7. Dose response of caffeine on young IMR-90 fibroblasts. Synchronized cultures were fed with MEM containing fetal calf serum without (○) or with caffeine: 0.1 mM (●), 1.0 mM (■), 5.0 mM (□). Samples were pulsed for 1 hr. with MEM containing 1.0 μCi/ml ^{3}H-thymidine at various intervals over a 36 hr. period. Aliquots of each sample were processed for cold trichloroacetic acid precipitable radioactivity and for measurement of protein content.

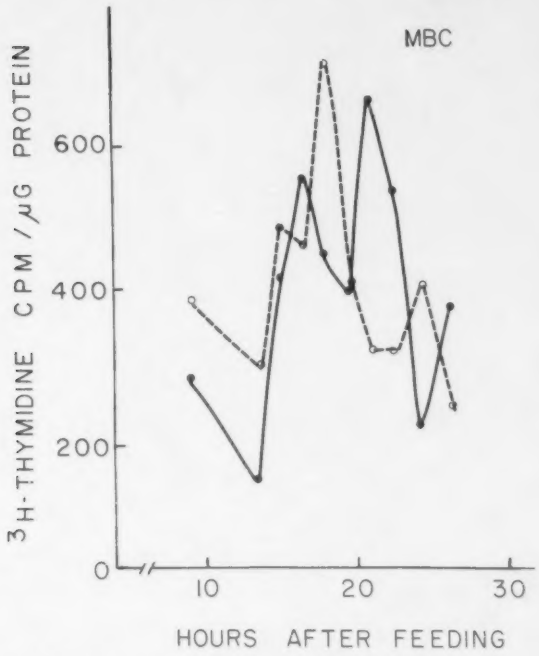

FIG. 8. Effects of caffeine on DNA synthesis of MBC. Synchronized cultures of MBC were fed with MEM containing fetal calf serum without (●) or with 0.1 mM caffeine (○). Over the course of 30 hrs., cells were pulsed for 1 hr. with MEM containing 1.0 μCi/ml ^{3}H-thymidine at various intervals. Aliquots of each sample were processed for cold trichloroacetic acid precipitable radioactivity and for measurement of protein content.

fibroblasts, both young and old, showed a marked depression in DNA synthesis. The younger cells were more sensitive to the doxorubicin, showing greater magnitude in the depression of thymidine incorporation seen than older cells.

SV-IMR90 (AG2804) cultures tested also showed an inhibition of DNA synthesis (Fig. 9). The magnitude of the depression lies between that of the young and old IMR90 fibroblast cultures tested. MBC, NA, 3T3 and SV-3T3 cultures all exhibited decreased DNA synthesis when exposed to doxorubicin (0.1 μg/ml). The magnitude of the depression for all four cell types was similar but much less than for the IMR90 fibroblasts and their SV40 virus transformed counterpart.

DISCUSSION

Various pharmacologic agents are capable of producing alterations in the cell cycle of synchronized cultures. This action may be a direct effect of the drug and correlate with its pharmacologic activity in vivo, or it may be a consequence of other metabolic changes. Cell culture provides a convenient method for examining these metabolic changes on a cellular and biochemical level.

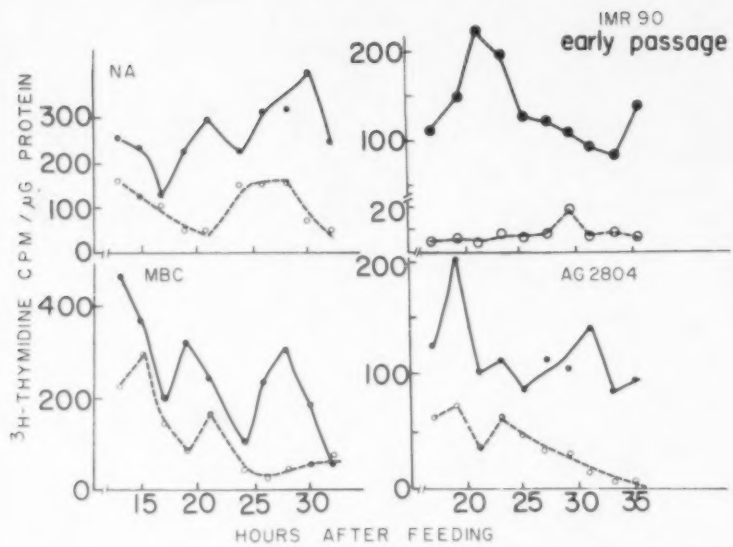

FIG. 9. Effects of doxorubicin on DNA synthesis. Synchronized cultures of NA, MBC, AG 2804 and IMR-90 early passage were fed with MEM containing fetal calf serum without (●) or with (○) 0.1 μg/ml doxorubicin. Samples were pulsed for 1 hr with MEM containing 1.0 μCi/ml ^{3}H-thymidine at various intervals throughout the cell cycle. Aliquots of each sample were processed for cold trichloroacetic acid precipitable radioactivity and protein content.

Normal human diploid fibroblasts exhibit many cellular and biochemical alterations upon exposure to steroidal anti-inflammatory agents (Cristofalo *et al.*, 1979; Grove and Cristofalo, 1977; Lamon *et al.*, 1982; Rosner and Cristofalo, 1979) including the stimulation of DNA synthesis observed in the work presented here. The young fibroblasts (IMR90) showed a dose-dependent increase in total DNA synthesis when exposed to the glucocorticoids, their older counterparts showing no stimulation. The decreased number of glucocorticoid binding sites in older cells (Rosner and Cristofalo, 1979) as well as the maintenance by hydrocortisone of a higher percentage of cells in the actively proliferating pool as suggested by Grove and Cristofalo (1977) may be attributed to the results observed here.

An effect on cell growth, both stimulatory and inhibitory, has been reported for a variety of cells other than human fibroblasts in culture upon exposure to the glucocorticoids (Fodge and Rubin, 1975; Ivaine *et al.*, 1980; Otto *et al.*, 1981; Ruhmann and Berliner, 1965; Young and Dean, 1980). We have observed here that upon transformation of IMR90 and WI38 fibroblasts by SV40 virus, the sensitivity to the steroids with respect to increased DNA synthesis is lost. This has also been reported elsewhere (Rosner and Cristofalo, 1979). This is even more apparent in 3T3 cells, which show a significant stimulation of thymidine incorporation into DNA even though their SV40 virus transformed counterparts show a marked inhibition. It appears that besides being cell, species and steroid specific, the effect of glucocorticoids on cells in culture is age and transformation dependent.

It has been reported that glucocorticoids cause a partial block in the G1 phase of the cell

cycle (Grove and Cristofalo, 1977; Young and Dean, 1980) and may account for some of the results seen here. Whether the G1 sensitive reactions are the same for both stimulation and inhibition of cell growth, and what cellular or environmental conditions are required for expression of these effects have yet to be determined.

Non-steroidal anti-inflammatory agents, such as aspirin, have also been shown to have an effect on the G1 phase of the cell cycle. It has been reported that these agents reversibly inhibit cell growth with an accumulation of cells arrested in G1 (Bayer and Beaven, 1979; Bayer *et al.*, 1979; Hial *et al.*, 1977). The cytostatic action of these drugs does not correlate with their ability to inhibit prostaglandin synthesis (de Mello *et al.*, 1980); however, it does correlate with their potency in vivo (Bayer *et al.*, 1980; de Mello *et al.*, 1980; Hial *et al.*, 1977). The dosages of aspirin that have been shown to inhibit DNA synthesis in cells in culture are approximately 20 times higher than the concentrations we have used in these studies.

An increase in labeled nucleotide incorporation into DNA was reported at concentrations of 1 or 2 mM aspirin (Hial *et al.*, 1977). When young IMR90 fibroblasts were exposed to concentrations 10-fold lower than this, a significant increase in labeled nucleotide incorporation was seen. Older IMR90 cells showed no alteration in DNA synthesis, exhibiting an age-dependent loss of sensitivity. The SV-IMR90 fibroblasts exhibited a marked decrease in DNA synthesis. Since SV-IMR90 fibroblasts parallel young IMR90 cells in certain age-related parameters (Kaji *et al.*, 1980), the difference in sensitivity and reaction to aspirin exposure may be related to the transformation of the cell.

The CNS stimulant amphetamine has been shown to cause disaggregation of rat brain polyribosomes in vivo as well as a dose-dependent inhibition of protein synthesis in vitro (Moskowitz *et al.*, 1975; Widelitz *et al.*, 1975; Baliga *et al.*, 1976; Saloman, 1978). In the studies presented here young WI38 and IMR90 fibroblasts were sensitive to 200 μM amphetamine exhibiting a significant decreased incorporation of labeled nucleotide into cold TCA precipitable material. Older fibroblasts showed no changes upon exposure to the same dose, exhibiting decreased sensitivity related to aging as has been reported here and by others for various drugs (Lamon *et al.*, 1982; Grove and Cristofalo, 1977). The SV40 virus transformed counterparts for these cells showed a significant increase in DNA synthesis. As with aspirin, this may be related to cytoplasmic or nuclear changes that occur as a result of transformation.

It has been shown that caffeine affects the genetic makeup of many cells in a variety of ways, including chromosomal aberrations and mutations, leading to cell death (Kihlman, 1977). These alterations have been seen with high doses greater than 5 mM of caffeine (Tatsumi and Strauss, 1979; O'Neill, 1979).

Caffeine has also been shown to have an effect on DNA synthesis. High doses of caffeine induce a significant depression in DNA synthesis in a variety of cells (O'Neill, 1979; Kihlman, 1977; Tatsumi and Strauss, 1979) and a dose-dependent competitive inhibition of thymidine kinase in Eschericia coli and human fibroblasts (Sandlie and Kleppe, 1980). Lower concentrations of caffeine have also been shown to induce an inhibition of DNA synthesis (Tatsumi and Strauss, 1974) as well as a reduction in the depression in DNA synthesis seen after treatment of mammalian cells with X-ray (Painter, 1980; Tolmach *et al.*, 1977).

In the studies presented here we have seen a dose-dependent depression in DNA synthesis in normal human fibroblasts at low concentrations of caffeine. This inhibition is also age sensitive since older normal fibroblasts were not inhibited to the same extent.

Synchronous cultures of HeLa S3 cells incubated with 1 mM caffeine, either continuously or during the *G1* phase only, showed a delayed entrance into the *S* phase (Tolmach *et al.*, 1977) suggesting that caffeine induces its effects on the cell during the *G1* phase. The delayed entrance into *S* phase of normal human fibroblasts and MBC seen here support this finding.

Although the evidence suggesting caffeine exerts its action in the G1 phase may explain the delayed initiation of DNA synthesis, it may not explain fully the changes in DNA synthesis seen. It has been shown that caffeine or its metabolites bind DNA (Kihlman, 1977; Robinson *et al.*, 1979) altering the conformation of chromatin (Painter, 1980; Lehmann, 1972), inhibiting chain elongation leading to synthesis of DNA in smaller units (Tatsumi and Strauss, 1979; Painter, 1980; Lehmann, 1972; Kihlman, 1977; Tolmach *et al.*, 1977), and increasing the number of replicon initiation sites (Tolmach *et al.*, 1977; Kihlman, 1977; Painter, 1980). These factors as well as cytoplasmic changes that may occur in *G1* may account for the results seen here.

Doxorubicin is an anthracycline antineoplastic agent. It has been shown to inhibit DNA synthesis in mouse ascites 6C3HD tumor cells in vivo (Tanaka and Yoshida, 1980) and *in vitro* in a variety of cells including L1210 mouse leukemia cells (Tanaka and Yoshida, 1980), CHO cells (Bhuyan *et al.*, 1980), Novikoff hepatoma ascites cells (Crooke *et al.*, 1978) and Chinese Hamster lung cells HT-1 (Waldes and Center, 1981). In our studies, all cell types tested showed marked depression of DNA synthesis. It has been shown to interact directly with chromatin. Doxorubicin induces DNA single-stranded regions, strand breaks, extremely fast sedimenting DNA and intercalation (Tanaka and Yoshida, 1980; Waldes and Center, 1981; Bhuyan *et al.*, 1980; Crooke *et al.*, 1978). It has also been shown to be a competitive inhibitor of DNA polymerases α and β subunits (Tanaka and Yoshida, 1980). It remains to be determined what is the primary mechanism of action of this drug.

In the studies presented here, we have tested drugs of varying pharmacologic activity *in vivo* for their effects on the cell cycle of different cell types *in vitro*. The use of synchronized cell cultures has enabled us to examine more closely the effect of these pharmacologic agents on DNA synthesis and compare normal with transformed as well as young with old cells. The results in this communication suggest that the use of tissue culture in the study of drugs may lead to the understanding of the macromolecular events at the cellular level.

SUMMARY

Effects of pharmacologic agents, such as steroidal and non-steroidal anti-inflammatory drugs, central nervous system stimulants, and antineoplastic agents were tested for their effects on macromolecular synthesis using synchronized cells induced by serum deprivation. It was found that 48 hours fasting for young and 60 hours fasting for old cells were necessary for human fibroblasts to be synchronized and that the initiation of cell synchronization was prompt in younger cells. Steroidal anti-inflammatory drugs strongly stimulated the uptake of thymidine into DNA in human and mouse fibroblasts but not in mouse brain and transformed cells. Aspirin, a non-steroidal anti-inflammatory agent, induced similar results. The CNS stimulant caffeine inhibited DNA synthesis in normal human and mouse fibroblasts but the magnitude of the depression was inversely proportional to the age of the cells. The inhibitory effect of caffeine on DNA synthesis was also

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seen in transformed cells. Amphetamine, also a CNS stimulant, stimulated nucleotide incorporation into DNA in transformed human fibroblasts but induced a slight depression in normal human fibroblasts. Doxorubicin, an anti-cancer agent acting on cellular DNA, caused a drastic suppression of macromolecular synthesis. These results suggest that studies of drugs using a tissue culture system may be used as a tool for the understanding of the macromolecular events at the cellular level.

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Experimental Gerontology, Vol. 17, pp. 359–364, 1982
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AGE CHANGES OF THE ISOELECTRIC POINTS OF NON-HISTONE CHROMOSOMAL PROTEINS FROM RAT LIVER IN THE pH RANGE 5 to 8

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(*Received* 2 *November* 1981)

INTRODUCTION

IT IS generally believed that the biological aging process has a genetic determinant, i.e., aging is a part of the developmental program inscribed in the genetic material, DNA (Morrow & Garner, 1979). Hence, the attention paid to chromatin and its components: DNA, histones, non-histone chromosomal proteins (NHC proteins).

It is also believed that chromatin activity is modulated by NHC proteins which are highly heterogenous, acidic and tissue-and species-specific (Barrett & Gould, 1973) being implicated in gene expression in general (Stein *et al.*, 1974) and in the control of transcription in particular (Wang & Kostraba, 1975; Nguyen *et al.*, 1975). NHC proteins undergo also covalent modifications like phosphorylation and acetylation (Liew & Gornall, 1975) which are in turn, modulated by calcium and estradiol in an age-dependent process (Kanungo & Thakur, 1979). In addition, there are reports about discrete variations with age among several specific proteins in rat liver nuclei of different ploidy (Garker & Brasch, 1979) and in human doploid fibroblasts (Dell'Orco *et al.*, 1978).

In literature there are some reports dealing with the isoelectric points of NHC proteins extracted from rat liver (Mac Gillivray & Rickwood, 1974; Gronow & Thackrah, 1978; Dayal *et al.*, 1979; Legraverend & Glazer, 1980) but the results can hardly be compared as the authors used a variety of extraction and isoelectric focusing methods. However, in our review on the literature, we did not find any relevant paper concerning isolectric points of NHC proteins in ageing rat liver. In the present article, we focused our attention on age-related changes in the isoelectric points of NHC proteins in the pH range 5–8.

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METHODS AND PROCEDURE

Animals

Each experiment was carried out on four groups of four Wistar male albino rats (young, 2–3 months; senescent, 24–28 months) maintained in a room at 22 ± 2°C on a 12 hours light/12 hours dark cycle. Animals were given commercial diet and water ad libitum.

Materials

Ampholines were received from LKB (Sweden); Nonidet P-40 was from Fluka (Switzerland) and phenylmethylsulfonyl fluoride was a generous gift from Serva (W. Germany); all others reagents were analytical grade.

Solutions used for preparation of NHC proteins contained 0.1 mM phenylmethylsulfonyl fluoride, for protease inhibition and 1 mM $NaMoO_4$, 50 mM NaF for phosphatase activity inhibition (Schmidt & Lindigkeit, 1977; Yeaman & Cohen, 1977).

Isolation of NHC proteins

The rats were killed by cervical dislocation, their livers were removed, washed with physiological saline, and weighed. The pooled livers were used for nuclei isolation by the method of Smuckler *et al.* (1976). The histones were extracted from purified nuclei by stirring the pellet in 25 ml of 0.25 N HCl at +2°C for three hours and washing the residual insoluble material with an additional volume of 5 ml of 0.25 N HCl. Acid insoluble residue was used for NHC proteins isolation, using the two-phase, chloroform-isoamyl alcohol, system of Chilina *et al.* (1976).

For sample preparation the residue was dissolved in 0.2 ml of 9.5 M urea, 2% (w/v) Nonidet *P*-40, 2% Ampholines (comprised of 1.6% pH range 5–8 and 0.4% pH range 3–10), 5% (v/v) β-mercaptoethanol, giving a protein concentration of 7.48 mg/ml for old rats and of 10.36 mg/ml for young rats. Protein concentration was determined after extensive dialysis of the sample against water, by the method of Lowry *et al.* (1951).

Isoelectric focusing

Isoelectric focusing gels were made in glass tubing (150 × 4 mm inside diameter) following essentially the technique described by O'Farrell (1975). The gels were run at 300 V for 36 hours at +4°C, then they were removed, fixed for one hour in trichloroacetic acid 12% (w/w)-4% sulfosalycylic acid, stained for two hours in 27% (v/v) isopropanol, 12% acetic acid, 0.04% Coomasie brilliant blue R-250, 0.5% $CuSO_4$ and destained in 12% isopropanol-10% acetic acid. The destained gels were scanned at 550 nm using a Carl Zeiss Jena densitometer. For pH gradient measurement, the gel was cut in slices of 2 mm which were placed in vials containing 2 ml of H_2O and shaken for ten min; the pH was measured on a Radiometer pH meter.

RESULTS

Fig. 1 is a photograph containing Coomasie brilliant blue stainable proteins from old rat liver (a) and young rat liver; (b) whereas, Fig. 2 and Fig. 3 represent the corresponding densitograms for old respectively young rats.

Twenty discrete bands were visualized for NHC proteins from old rats (Fig. 1a) which appeared as peaks or shoulders on the densitometer tracing (Fig. 2). For young rats, about 25 bands were visualized on Fig. 1b; these appeared as peaks or shoulders on the densitometer tracing in Fig. 3.

On the densitograms, four areas were formally separated (Table 1) each corresponding to a pH range: I) 5.80–6.10; II) 6.25–6.42; III) 6.80–7.00; IV) 7.20–7.35 for young rats, and I) absent; II) 6.35–6.75; III) 6.80–7.00; IV) 7.12–7.40 for old rats. From Table 1 and Fig. 1 one can easily observe a group of proteins from young rats having the pH range 5.80–6.10 which is not present in old rats. The intensity of the peaks is relatively uniform for young animals; whereas, for old animals there is a distinct peak in the pH range 6.35–6.75. Due to Ampholines the background of the gels is relatively high.

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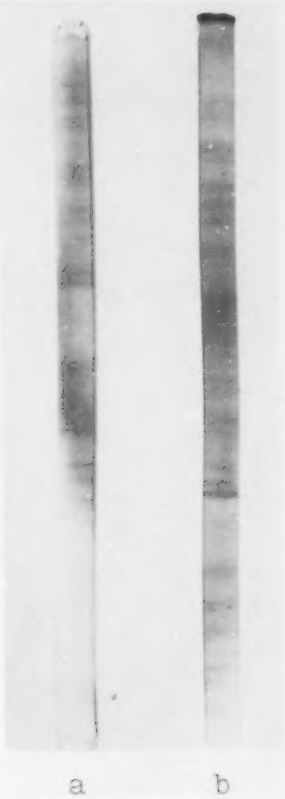

FIG. 1. Isoelectric focusing separation of NHC proteins from old rat liver (a) and young rat liver (b). 150 μg of proteins were loaded onto the gels and isoelectric focusing was run for 36 hours at 300 V at +4°C. For other experimental details see the text. The top of the gel represents the cathode end.

DISCUSSION

During experiments we tried to protect protein degradation by proteases by including phenylmethylsulfonyl fluoride (0.1 mM) in all homogenizing media, and at the same time we added Na_2MoO_4 (1 mM) and NaF (50 mM) for phosphatase activity inhibition. In addition, we avoided in isolation procedures, extreme values of pH which could lead to breakdown of P-O and P-N linkages and to other hydrolytic processes.

From all described techniques for gel isoelectric focusing, we chose the method of O'Farrell which was found to be the best choice by many authors since it has appeared in 1975. In our hands the method gave a good resolution and a satisfactory reproducibility. From data presented under Results, we established that there was a protein fraction in the pH range 5.80–6.10 not present in old animals, the rest of fractions designated by the II, III and IV areas being remarkable similar in respect to pH range.

The separation of NHC proteins by means of isoelectric focusing is primarily associated

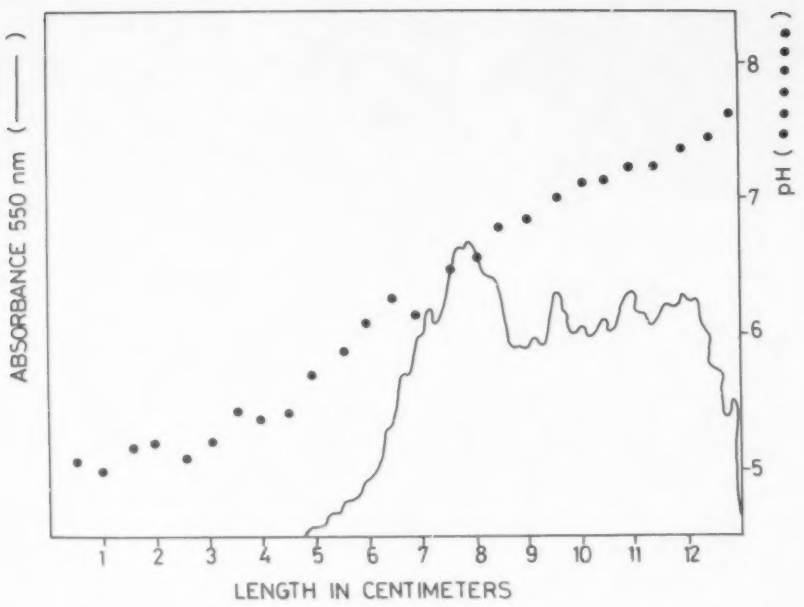

FIG. 2. Densitometer tracing of NHC proteins from old rat liver separated on analytical isoelectric focusing gel.

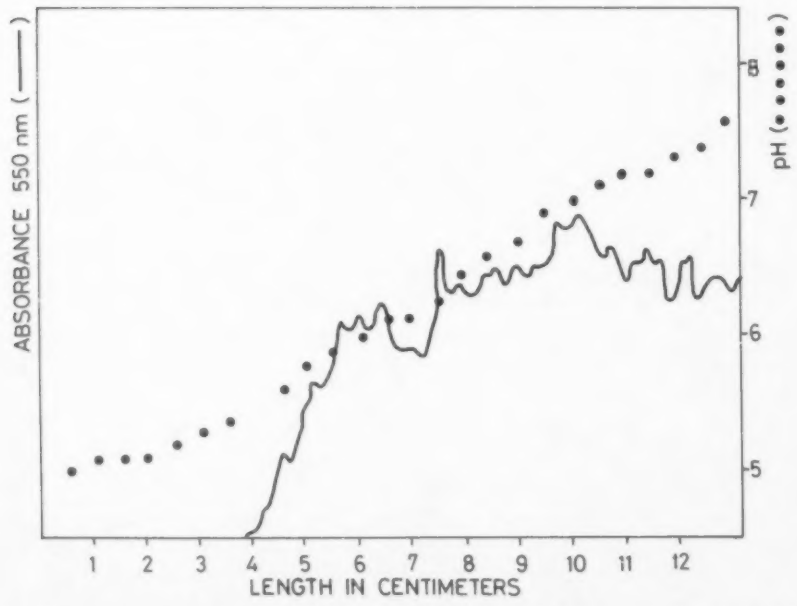

FIG. 3. Densitometer tracing of NHC proteins from young rat liver separated on analytical isoelectric focusing gel.

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TABLE 1. THE pH RANGE AREAS CORRESPONDING TO NHC PROTEINS GROUPS FORMALLY SEPARATED ON THE DENSITOGRAMS DERIVED FROM ISOELECTRIC FOCUSING GELS.

	Area	pH range
Young rats	I	5.80–6.10
	II	6.25–6.42
	III	6.80–7.00
	IV	7.20–7.35
Old rats	I	–
	II	6.35–6.75
	III	6.80–7.00
	IV	7.12–7.40

with their global electric charge which in turn, depends on acidic aminoacids/basic amino acids ratio. However, the global charge is modified by well known post-translational modifications: phosphorylation, acetylation, ADP-ribosylation, deamidation, terminal peptide cleavage, glycosylation. Consideration of only one of these modifications could lead to puzzling results: incorporation of (^{32}P) labelled phosphate into the 0.35 M NaCl soluble NHC proteins was higher in the alkaline region of the isoelectric focusing gel for a ratio Asp + Glu/Lys + His + Arg = 1.93 (Dayal *et al.*, 1979). In addition, the preparations of NHC proteins often contain a small amount of tightly bound nucleic acids (Bhorjee & Pederson, 1976) which lower the isoelectric points of the associated proteins.

Unfortunately, there are only a few complete studies concerning some of the modifications indicated: Liew & Gornall (1975) reported that acetylation of phenol soluble nuclear acidic proteins was increased to 250% and phosphorylation to 138% in liver of old mice as compared to young mice, whereas Kanungo & Thakur (1979) found that acetylation and phosphorylation were decreased in brain of aged female rats.

The validity of the results reported by Liew & Gornall is questionable as Thakur *et al.* (1978) and Kanungo & Thakur (1979) showed that phosphrylation and acetylation are under hormonal control.

The patterns of densitograms shown in Fig. 2 and Fig. 3 are due probably to the global electric charge resulting from the post-translational modifications indicated and to nucleic acids impurities stainable with ethidium bromide (results not shown as there were no significant differences between old and young rats). The presence of protein fraction I on gels from young rats, was neither an experimental artifact as it appeared in each experiment, nor was it due to a lower protein concentration loaded onto the gels from old rats as it did not appear at even twice the concentration of proteins from young rats.

Conclusions

The NHC proteins isolated from histone depleted nuclei and separated on analytical isoelectric focusing gels in the pH range 5–8, showed a discrete, age-dependent distribution. The factors which led to an age-dependent distribution could be numerous but we outlined the importance of post-translational modifications as modulators of the global electric charge of NHC proteins.

SUMMARY

Non-histone chromosomal proteins were extracted from purified nuclei of young (2–3 months) and old (24–28 months) rat liver by a two-phase partition method (chloroform:-isoamyl alcohol, 24:1) after histone depletion with 0.25N HC1. The proteins were subjected to analytical isoelectric focusing in the pH range 5–8. On the densitograms derived from Coomasie brilliant blue R-250 stained gels, there were formally four areas separated having the following pH ranges: I) 5.80–6.10; II) 6.25–6.42; III) 6.80–7.00; IV) 7.20–7.35 for young rats, and I) absent; II) 6.35–6.75; III) 6.80–7.00; IV) 7.12–7.40 for old rats.

Hence, the main result is the absence of a group of proteins from old rats having the pH range 5.80–6.10. The factors involved in protein separation by isoelectric focusing, like time-dependent post-translational charge modifications: phosphorylation, acetylation, ADP-ribosylation, deamidation, terminal peptide cleavage, glycosylation, and DNA-like contaminants are discussed.

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Experimental Gerontology, Vol. 17, pp. 365–373, 1982
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A NON-STEROIDAL GAMETIC FACTOR LINKED TO DNA MODULATES δ-AMINOLEVULINIC ACID SYNTHASE INDUCIBILITY ACTING ON LIVER TRANSCRIPTIONAL AND TRANSLATIONAL PROCESSES

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(*Received* 20 *November* 1981)

Abstract—The effect of an acidic factor of low molecular weight (about 1,000 daltons), extracted from bovine spermatozoan DNA, on the inducibility of δ-aminolevulinic acid synthase by ethanol during aging in rat has been examined. The increased enzyme inducibility in 600-day old rats is supported by stimulation of transcriptional and translational processes; on the contrary, in 30-day old rats, the higher enzyme values induced by ethanol are significantly decreased after factor treatment. The active factor is strongly DNA-bound in the native spermatozoan DNA. This would imply a possible role in regulating gene expression *in vivo*.

INTRODUCTION

Previous studies (Casellato *et al.*, 1980) have shown that a non-steroidal factor, extracted from male gametes *in toto*, is able to restore the inducibility of some enzymes (NADPH-oxidase, tyrosine aminotransferase and δ-aminolevulinic acid synthase) in liver of old rats. The reported data have suggested that the gametic factor influences, in conditions of impaired response to exogenous stimuli, some liver cell functional properties.

In order to assess the possible interference of the gametic factor with liver metabolism, and taking into account that this factor is probably strongly linked (>10 kcal mole^{-1}) to spermatozoan DNA (Guglielmi *et al.*, 1978), a new selective method for the extraction and purification of the active fraction from isolated nuclear chromatin was performed.

The gametic factor obtained in this way was administered to young and old rats, and the following hepatic parameters were evaluated: δ-aminolevulinic acid synthase (ALA-synthase, 2.3.1.37) activity, nucleic acid and protein contents, DNA replication, DNA transcription and RNA translation processes.

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MATERIAL AND METHODS

Animals

Male Sprague-Dawley O.R. rats of 30 and 600 days of age (average body-weight 90 g and 575 g, respectively), were housed at 23° ± 1° C, 60% relative humidity and under a controlled illumination programme that provided a 12 hrs. light period from 7 a.m. and 7 p.m.

Protein content

Protein determination of fractions isolated from DNA was performed in the ninhydrin test, according to Moore and Stein (1954), after alkaline hydrolisis in order to avoid interferences due to buffer, using L-leucine as standard.

Extraction and purification of the gametic factor

All the reported steps were carried out in the cold (+2° C), unless otherwise stated. The whole extraction and purification of the gametic factor was done as previously described (Casellato *et al.*, 1980), starting not from the male gamete homogenate *in toto* but from nuclear chromatin isolated from bovine spermatozoa treated according to Borenfreund *et al.* (1961), with minor modifications. Thus, 100 ml of bovine semen, containing about 2×10^{11} spermatozoa, were centrifuged at 8,000 × g for 20 min, and the sperm pellet washed twice with 0.15 M sodium chloride – 0.015 M sodium citrate solution (SSC) pH 8.1, once with 95% ethanol, then twice with 1:1 (v:v) mixture of ethanol and diethyl ether.

To the dried lipid-free sample was added SSC (200:1, v:v), containing 2% β-mercaptoethanol, and the mixture was then incubated for 2 hrs. at 20° C under gentle stirring. Trypsin (from porcine pancreas, 10,000-13,000 BAEE units/g, DE.BI., Italy) was then added, 100 mg for 1 g dried sperm pellet, and the incubation continued at 20° C for 2–3 hrs. at constant pH (8.1).

After centrifugation at 13,000 × g for 30 min, the enzymatic treatment (50 mg trypsin for 1 g) was repeated twice. The three supernatants were collected, then precipitated with two volumes of 95% ethanol. The fibres which formed were removed with a glass rod, washed at first with 66% ethanol, then with 95% ethanol and dried under vacuum.

The crude DNA (0.98 g), suspended in water, was brought with dilute sodium hydroxide solution at pH about 10, precipitated with two volumes of 95% ethanol, left overnight, then centrifuged at 13,000 × g for 30 min.

The microfibrillar pellet, washed with 95% ethanol and dried (0.552 g, equivalent to 0.445 g DNA, as calculated from absorbance at 259 nm), was solubilized in water. To the solution was added one volume of 0.4 M ammonium acetate-ammonia buffer pH 9.5, then precipitated under vigorous stirring with two volumes of 95% ethanol, and left overnight.

After centrifugation at 13,000 × g for 30 min the supernantant was evaporated to small volume under vacuum at 30°–35° C. The concentrated solution (pH 6.7) was ultrafiltered through a membrane with a porosity less than 5 nm (Sartorius-Membranfilter 12136, molecular weight cut off < 10,000 daltons), then it was lyophilized three times.

The active fraction (DS1-B, 0.995 mg) contains small peptides (molecular weight about 1,000 daltons) as the major component. Nucleic acid material is absent: the U.V. absorption of a solution in water does not exhibit any maximum at 259 nm.

The presence of free steroids or their conjugates in the DS1-B fraction was excluded (before and after enzymatic digestion with glucuronidase and sulphatase) by radioimmunassay for testosterone, 5α-dihydrotestosterone, progesterone and 17β-estradiol (< 10 pg for 10 μg fraction DS1-B, equivalent to about 2×10^9 spermatozoa or 1 ml total semen).

Treatment

ALA synthase activity was induced with 30% ethanol in saline i.p. (300 mg ethanol/100 g b.w.), 4 hrs. before kill, as described by Paterniti *et al.* (1978). When treated with the gametic factor, rats received subcutaneous injections twice a day for 3 days of 2 ml/kg b.w. saline, containing the purified active extract at two dose levels, equivalent to 1 and 10 μg of protein, and corresponding to about 2×10^8 spermatozoa (0.1 ml semen) *pro die*/kg b.w. and about 2×10^9 spermatozoa (1 ml semen) *pro die*/kg b.w. Control-uninduced and control-ethanol induced rats received the same volume of saline.

Preparation of liver extracts and ALA-synthase, nucleic acid and protein contents, DNA replication, DNA transcription and RNA translation determination.

All the labelled compounds were purchased from Radiocheminal Center, Amersham, U.K. Rats were killed by decapitation. The livers were quickly removed, washed in cold saline and weighed. Each liver divided in three

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parts. The first was homogenated in saline (1:5, w:v) and used for DNA (Setaro and Morley, 1977), RNA [Mesbaum, 1939 (modified)] and protein content (Lowry *et al.*, 1951) determinations.

A second part was homogenated in 10 mM Tris-HCl buffer (pH 7.4), containing 0.154 M sodium chloride (1:5, w:v), and aliquots of the homogenate were used for ALA-synthase assay, as described before (Caseilato *et al.*, 1980), evaluating the incorporation of [2,3-^{14}C] succinic acid (s.a. 17.5 mCi/mmol). Another part of liver was used for the isolation of nuclei and postmitocondrial supernatant. Replication and transcription were determined measuring the incorporation of [methyl-^{3}H] thymidine 5'-triphosphate (ammonium salt, s.a. 40–60 Ci/mmol) and [4-^{14}C] uridine 5'-triphosphate (ammonium salt, s.a. 40–60 mCi/mmol), respectively, in liver nuclei DNA and RNA, according to Burke *et al.* (1979) and Voets *et al.* (1979).

Protein synthesis was performed evaluating L-[U-^{14}C] leucine (s.a. >300 mCi/mmol) incorporation in postmitocondrial supernatant according to Ragnotti and Aletti (1975).

Samples were counted in a Packard Tri-Carb scintillation counter, using Pico-Fluor™ 30 as scintillator solution.

RESULTS

In Fig. 1 the basal and ethanol-induced activity of ALA-synthase in 600-day and 30-day old rats, without or with gametic factor treatment at two doses, are reported. It is evident that the gametic factor is able to cause, with a dose-dependent correlation, a significant increase in enzyme levels in old rats, though it decreases enzyme levels in young rats. The gametic factor alone does not show any effect in uninduced rats.

Table 1 reports the results obtained about DNA, RNA, protein content in liver homogenate and DNA replication in isolated liver nuclei, both in uninduced, induced and induced-treated rats. DNA transcription in isolated liver nuclei and protein synthesis in liver postmitocondrial supernatant are reported in Figs. 2 and 3, respectively.

From the data here reported there is evident a slight decrease of RNA and protein content in 30-day-old induced rats, treated with 10 μg of gametic factor (17% and 14%, respectively), in comparison with the induced-controls. On the contrary, in 600-day old rats, RNA and protein contents are slightly increased (6% and 18%, respectively). No significant differences are present in DNA content and in DNA replication. Transcription and translation processes appear markedly influenced by the administration of the active factor. In fact, ethanol-treated aging rats show a decrease of transcription and protein synthesis compared to controls; after the treatment with the gametic factor, a stimulation of transcription over that in induced controls is shown (+44% and +53% with 1 μg and 10 μg treatment, respectively).

The inhibition of protein synthesis by ethanol (induced-controls −41% over that in uninduced-controls) is less marked (−26% at dose 1 μg and −14% at dose 10 μg over that in uninduced controls, equivalent to an increase of 25% and 45% when compared to values of ethanol induced-controls).

DISCUSSION

The highly significant increase, evident after gametic factor treatment, in ALA-synthase activity of ethanol induced aging rats (this activity is strongly reduced in ethanol induced old animals compared to young ones), shows the capacity of the gametic factor to influence *in vivo*, in condition of impaired response, the activity of this enzyme. Moreover in ethanol induced old rats, after gametic factor treatment, an evident dose-dependent increase of RNA and protein synthesis is present.

These data suggest that the gametic factor is able to promote ALA-synthase activity in old rats by stimulating transcriptional and translational processes and, consequently, increasing the synthesis of new enzyme molecules. This would mean that the significant decrease in the inducibility of ALA-synthase in old rats is associated with a decreased effi-

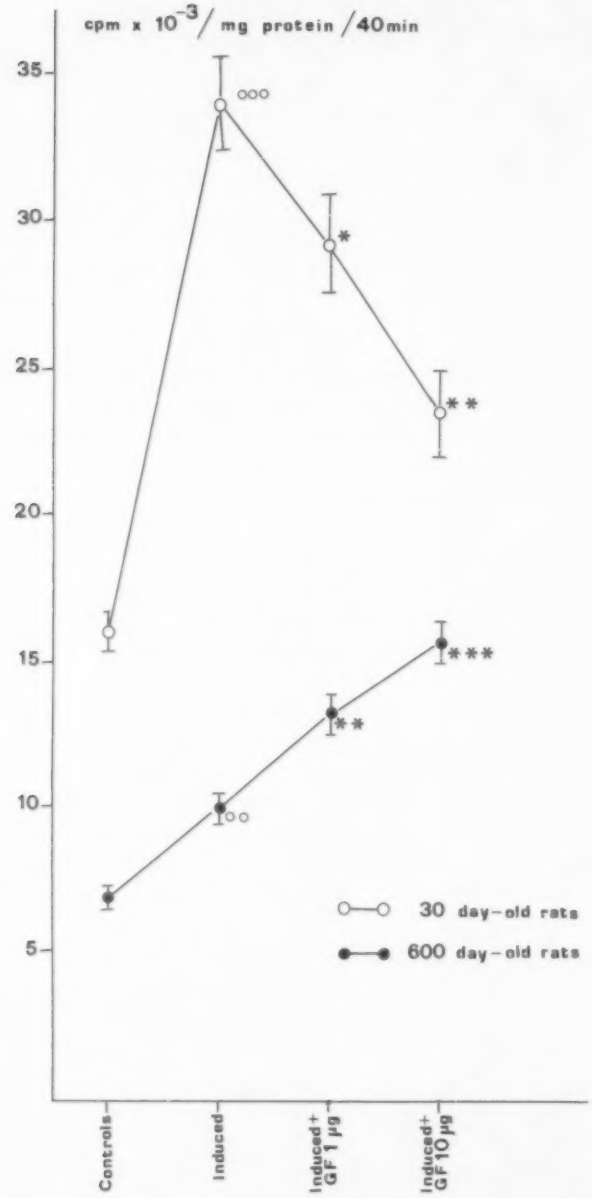

FIG. 1. Effect of the gametic factor (GF) on ALA-synthase in ethanol-induced rats, by evaluating the incorporation of [2, 3-^{14}C] succinic acid. Means ± s.e.m. Each group consisted of 7 animals. $^{*}P = 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. ethanol-induced rats; $^{\circ\circ\circ}P < 0.001$ vs. saline controls.

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TABLE 1. EFFECT OF THE GAMETIC FACTOR (GF) ON DNA, RNA, PROTEIN CONTENTS AND REPLICATION IN LIVER OF ETHANOL-INDUCED RATS. MEAN ± S.E.M. EACH GROUP CONSISTED OF 7 ANIMALS.

	30-day-old				600-day-old			
Determinations	Controls	Induced	Induced + GF 1 μg	Induced + GF 10 μg	Controls	Induced	Induced + GF 1 μg	Induced + GF 10 μg
DNA (mg/g wet liver)	3.01 ± 0.12	3.20 ± 0.20 (+6%)[a]	3.10 ± 0.14 (−3%)[b]	2.99 ± 0.13 (−6%)[b]	2.79 ± 0.13	2.76 ± 0.09 (−1%)[a]	2.90 ± 0.14 (+5%)[b]	2.87 ± 0.13 (+4%)[b]
RNA (mg/g wet liver)	9.90 ± 0.81 (+19%)[a]	11.82 ± 1.33° (+19%)[a]	9.71 ± 0.89* (−18%)[b]	9.78 ± 0.74* (−17%)[b]	8.50 ± 0.72	8.52 ± 0.56 (−)[a]	8.75 ± 0.63 (+3%)[b]	9.01 ± 0.70 (+6%)[b]
Protein content (mg/g wet liver)	215 ± 15.2	233 ± 17.4 (+8%)[a]	208 ± 12.1* (−11%)[b]	200 ± 10.9* (−14%)[b]	324 ± 20.2	293 ± 16.5 (−10%)[a]	336 ± 17.2* (+15%)[b]	346 ± 16.1* (+18%)[b]
Replication (cpm/μg DNA)	143 ± 9.2	150 ± 10.4 (+5%)[a]	146 ± 8.3 (−3%)[b]	149 ± 10.6 (−1%)[b]	151 ± 8.4	157 ± 9.9 (+4%)[a]	155 ± 7.9 (−1%)[b]	160 ± 8.5 (+2%)[b]

[a] The percentage of increase versus saline-uninduced controls.
[b] The percentage of increase versus ethanol-induced controls.
* $P = 0.05$ vs. induced controls.
° $P = 0.05$ vs. saline controls.

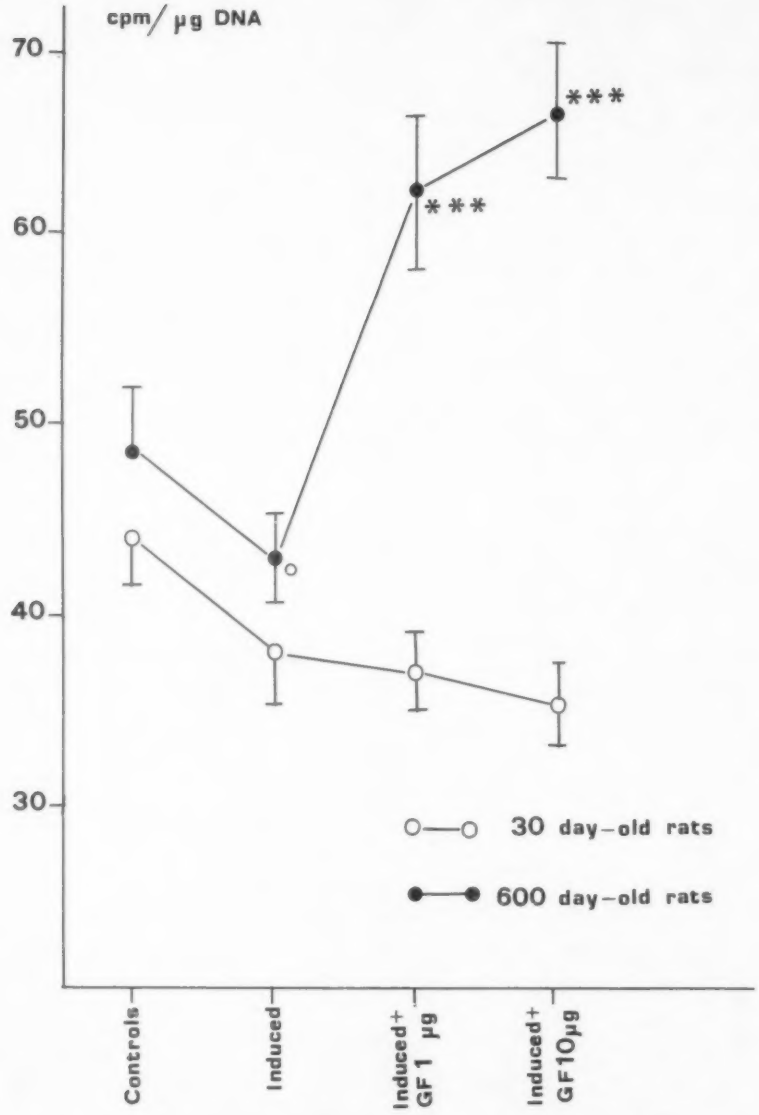

FIG. 2. Effect of the gametic factor (GF) on isolated nuclei transcription in ethanol-induced rats, by evaluating the incorporation in RNA of [4-^{14}C] uridine 5'-triphosphate. Means ± s.e.m. Each group consisted of 7 animals. ***$P < 0.001$ vs. ethanol-induced rats; °$P = 0.05$ vs. saline controls.

ciency of enzymatic protein synthesis, which is a feature of senescent animals. In fact, protein synthesis is decreased with advancing age in rat liver (Buetow and Gandhi, 1973; Comolli *et al*, 1972 and 1977; Comolli, 1973) and also the inducibility of liver enzymes is impaired in this condition (Adelman, 1972).

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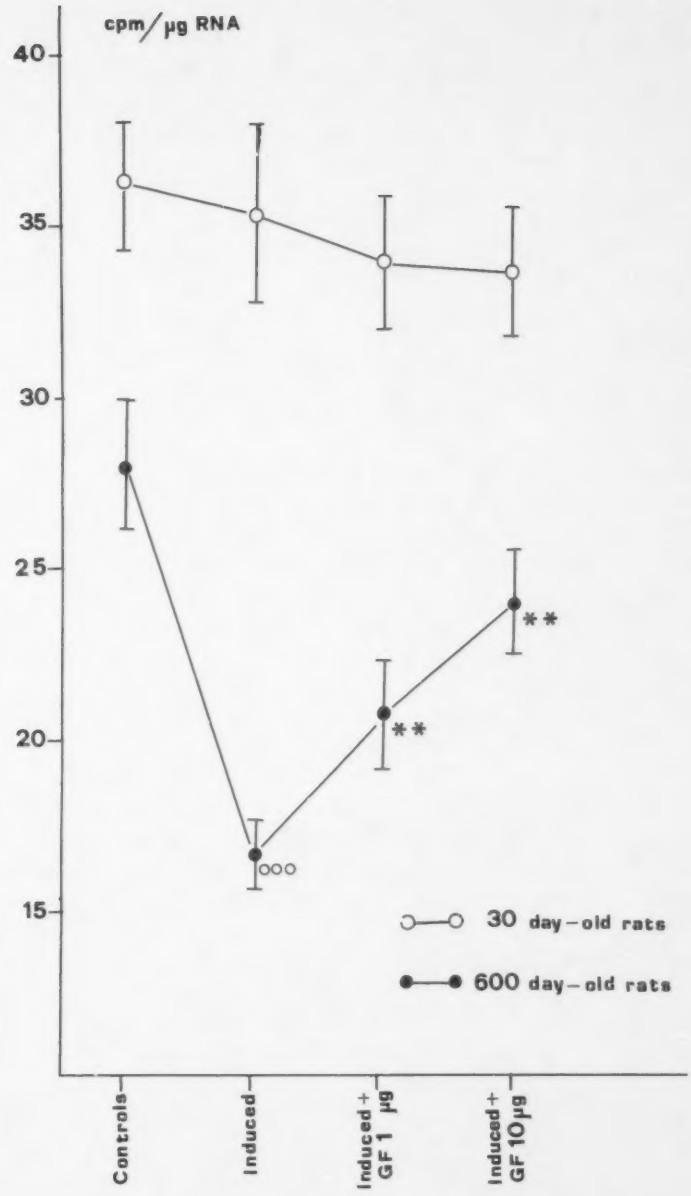

FIG. 3. Effect of the gametic factor (GF) on postmitocondrial supernatant protein synthesis in ethanol-induced rats, by evaluating the incorporation of L-[-U-^{14}C] leucine. Mean ± s.e.m. Each group consisted of 7 animals. **P < 0.01 vs. ethanol-induced rats; °°°P < 0.001 vs. saline controls.

These observations are consistent with results previously reported (Fachini *et al.*, 1977), showing that the activity of the gametic factor occurs only in condition of impaired response to exogenous stimuli, particularly observed in many biochemical functions which commonly undergo senile modifications, e.g., the lipoprotidaemic spectra (Amici *et al.*, 1974; Gianfranceschi *et al.*, 1974), and more generally in conditions related to slowed down cytodynamics such as delayed wound healing and repair of bone fractures (Teneff, 1970) or senile cataract in dog (Lugaro *et al.*, 1980) and in man (Lugaro *et al.*, 1982).

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It is possible that the gametic factor, as native chromatin constituent, is involved in the arrangement of the chromatin structure itself. By the method of extraction here reported, we put forward the hypothesis that the active factor is strongly DNA-bound in the native spermatozoan DNA. The interaction between DNA and the active factor is also demonstrated by the melting profile of DNA in heat denaturation experiments carried out according to Gianfranceschi *et al.* (1977). The T_m (melting or transition temperature) of control DNA (78.5° C) was considerably lower than the T_m (83.0° C) of DNA complexed with a saturant dose of the active factor (about 1:6 ratio gametic factor: DNA) (Lugaro *et al.*, in press).

The strength of the interaction between DNA and the gametic factor, its acidic nature and the aminoacids present in purified fraction (a tentative and qualitative analysis of the purified gametic factor shows that it lacks arginine and histidine; there are present glutamic and aspartic acids, alanine, serine, valine, isoleucine, glycine and perhaps lysine) allow us to exclude the possibility that the active factor of low molecular weight (about 1,000 daltons) is a known histone or histone fragment. It is known that the resolution of non-histone proteins has proved particularly difficult because of the high affinity for DNA (Bloom and Anderson, 1977).

Furthermore, the chemical and physical data concerning the interaction between DNA and the active factor show that it can be dissociated from DNA by ultracentrifugation of solution of high ionic strength (4 M CsCl) but not by an acid solution (0.25 M HCl): thus refuting once more the possibility that the gametic factor might be a histone fragment.

Since the inducibility of ALA-synthase is altered during aging and also in the majority of rat hepatomas, this gametic factor might possibly enter in the regulation mechanism of enzyme activity in the neoplastic cells also. Hillar and Przyjemski (1979) stated that loss or decreased levels of similar factors, present in and extractable from liver, thymus and spleen DNA, are responsible during the promotion of carcinogenesis for incontrolled gene expression. It is reasonable to hypothesize also that the restoration of inducibility of NADPH-oxidase and tyrosine aminotransferase (Casellato *et al.*, 1980) in liver of aging rats is identically correlated to the same increases of transcription and protein synthesis processes.

SUMMARY

The effect of a low molecular weight non steroidal gametic factor, extracted from purified bovine spermatozoan DNA, on the inducibility of δ-aminolevulinic acid synthase by ethanol during aging in rat has been examined. The results here reported show that this factor is able to influence the inducibility of liver enzyme in condition of impaired response to exogenous stimuli.

The increase δ-aminolevulinic acid synthase inducibility in 600-day old rats is supported by stimulation of transcriptional and translational processes and, consequently, by in-

crease of the synthesis of new enzyme molecules. In 30-day old rats, the higher values induced by ethanol are significantly decreased after gametic factor treatment. The active factor, a molecule of acidic nature and of low molecular weight (about 1,000 daltons), is presumably strongly DNA-bound in the native spermatozoan DNA. This would imply a possible role in regulating gene expression *in vivo*.

Acknowledgements—This work was supported by the Programma Finalizzato 'Biologia della Riproduzione', C.N.R., Italy.

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Experimental Gerontology, Vol. 17, pp. 375–381, 1982
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0531-5565/82/050375-07$03.00/0
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ANTIGENIC DETERMINANTS ON LYMPHOCYTES FROM YOUNG AND OLD C57BL/6 MICE

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(*Received* 1 *December* 1981)

INTRODUCTION

THE DECLINE in immune function with age may be due to alterations in lymphocytes themselves as well as to changes in their number and subpopulations (Makinodan and Kay, 1980). Alterations in lymphocytes of aged animals leading to immunological responses with syngeneic young animals have been reported. Gozes *et al.* (1978) found that the injection of spleen cells from old mice into the footpads of syngeneic young or old mice caused significant enlargement of the popliteal lymph node of the recipient. Callard *et al.* (1979) obtained a young anti-old serum by injecting young mice with cells from syngeneic old mice. They also investigated the mixed lymphocyte reaction (MLR) of spleen cells from young and old mice in all combinations. All their old mice responded to young and nearly all the young mice responded to old, but in no case was there a young → young or old → old response. However, in a similar study, Moore *et al.* (1981) found that fewer than half of the old animals stimulated cells from young mice. No response of old to young was observed. Also in this study, spleen cells from young mice injected into syngeneic old animals produced significant splenomegaly in half of the old recipients.

The purpose of our study was to further investigate the extent of the differences between lymphocytes from young and old mice, especially to determine whether there are new or altered antigens present on cells in old animals. If such antigens exist, they would have considerable signficance not only in terms of the changes in immune function with age but as a possible marker of aging.

We have studied the response of lymphocytes from young and old female C57BL/6 mice with 3 techniques: the mixed lymphocyte reaction, cytotoxicity of sensitized lymphocytes from young mice toward lymphocytes from old mice, and the presence of antibody in sera of young mice immunized with lymphocytes from old mice.

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METHODS AND PROCEDURE

Animals

Female C57BL/6 mice, aged 2 to 31 months, were obtained from Charles River Breeding Laboratories (Wilmington, MA). The old animals were aged at Charles River facility under a National Institute on Aging contract, except where noted. Mice were examined before and after sacrifice and those with any gross pathology were not used in the experiments.

Mixed lymphocyte reaction

Young and old mice were killed by ether and spleens were removed. Single-cell suspension of spleen was prepared under sterile conditions. Young mice were 2 to 6 months old. Old mice were 24 to 31 months old. Spleens were teased in saline with forceps to disperse cells. The cell suspension was then passed through a 23-gauge needle, centrifuged and washed with saline three times. Cells were suspended in RPMI 1640 with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone (all from GIBCO, Grand Island, NY) and 5×10^{-5} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY) (TC medium). Stimulator cells were exposed to 1500 rads radiation with a Picker Vanguard 280 kilovolt X-ray machine. Cells were cultured in all possible one-way responder → stimulator combinations: young → young, young → old, old → young and old → old. Stimulator and responder cells (8×10^6 of each) in a total volume of 0.2 ml were dispensed into round-bottomed-well microtiter plates (Linbro Scientific, Hamden, CT). All cultures were performed in triplicate. Controls consisted of responder cells cultured with stimulator cells from the same mouse. Plates were incubated at 37°C in a 5% CO_2 water-saturated air incubator. Following 48 hrs. of incubation, 0.4 μCi ^{3}H-thymidine (0.1 μCi/μl, specific activity 75 Ci/mM) was added to each well. Following 24 hrs. thymidine pulse, the cultures were harvested with a multiple automatic sample harvester onto glass-fiber filters. The filters were dried and counted in 2.5 ml scintillation fluid in a Beckman LS-8000 liquid scintillation counter. Experiments were performed with groups of 4 young and 4 old mice (three times) or 3 young and 3 old mice (twice), a total of 18 young and 18 old animals. Mice within each group were from the same cohort. Additional MLRs were performed with the addition of 4% polyethylene glycol (PEG, molecular weight 6000, Sigma Chemical Co., St. Louis, MO) to the culture medium. In the first experiment 4 young and 4 old mice were used. PEG was added to all cultures. In a second experiment, 2 young and 2 old mice were used with and without the addition of PEG to cells from the same animals.

Analysis of MLR data

Responses of the MLRs without PEG were grouped according to the type of stimulator-responder combination. The number of significant responses above controls ($P < 0.05$) was determined by a one-tailed Dunnett's test. Differences among the 4 groups of stimulator-responder combinations were analyzed by a X^2 test.

Cytotoxicity of sensitized young lymphocytes

This assay involved sensitizing lymphocytes from young (5–6 month-old mice) against old (27–31 month-old mice) lymphocytes by culturing *in vitro* in a MLR. Cytotoxicity to old cells was then determined by the ^{51}Cr-release assay. We first confirmed the validity of this technique, which was based on the method of Spits *et al.* (1981), and identified optimum conditions for the assay using C57BL/6 target cells and sensitized BALB/c effector cells. Spleens were removed from a young and old animal and the cells prepared as described above. The old cells were exposed to 1500 rads. Cells were cultured in TC medium in a 25 cm^2 tissue culture flask at a density of 5×10^6 cells/ml from young mouse and an equal number of cells from old mouse. The cytotoxicity assay was performed after 5 days culture. Two days prior to the assay the spleen was obtained from an old mouse. Single cell suspension of spleen was prepared, washed and the red blood cells were lysed with 0.83% NH_4Cl-tris buffer. Cell suspension was adjusted to contain 2×10^6 cells/ml in TC medium with 10 μg/ml phytohemagglutinin (PHA). After 48 hrs. incubation, viable cells were counted by trypan blue exclusion method and labelled with ^{51}Cr. One million cells in 100 μl RPMI 1640, 200 μCi of $Na_2\ ^{51}CrO_4$ and 2% fetal calf serum were incubated at 37°C for 45 min. Cells were washed three times with saline, counted and suspended in TC medium at a density of 10^4 cells/100 μl. One hundred microliters of effector cells from the MLR was added to 10^4 target cells in a flat-bottomed microtiter plate at effector:target ratios of 200:1, 100:1, 50:1 and 25:1 in triplicate. After incubation for 6 hrs, the plate was centrifuged (800 × G, 5 min.) and 100 μl of supernatant from each well was counted in a Beckman 4000 Gamma counter. Maximum chromium release was determined by freezing and thawing cells several times. Control effector cells from nonsensitized young mice were processed in an identical manner. A total of 5 old mice and 5 young mice were used for the MLRs. In one experiment, phorbol myristic acetate, which has been found to enhance cytotoxicity (Rosenstreich and Mizel, 1979) was added to the MLR at a concentration of 10 ng/ml.

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Young anti-old serum

Young anti-old lymphocyte antiserum was prepared according to the method of Callard *et al.* (1979). Pooled spleen and lymph node cells from old mice (24–30 months old, aged in our laboratory) were injected intraperitoneally into 10 young (3 month-old) mice. Each young mouse received 10^7 cells once a week. The first and subsequent odd-numbered injections were given with Freund's complete adjuvant. The second and subsequent even-numbered injections consisted of viable cells. A total of 8 injections were made over 8 weeks. Serum was collected one week after the last injection.

Cytotoxicity of young anti-old serum

Complement-dependent cytotoxicity was determined by the method of Boyse *et al.* (1964). One hundred microliters of serum was added to 10^5 target cells and incubated for 1 hr. at 37°C. The cells were washed three times with medium 199 containing 10% fetal calf serum. Cells were then suspended in 25 μl of guinea pig complement (1:10 dilution) and incubated for 30 min. at 37°C. Twenty-five microliters of 0.33% trypan blue was added and the dead and viable cells were counted in a hemocytometer. Normal young mouse serum and medium 199 plus 10% fetal calf serum were used in place of the antiserum as controls. Target cells were from spleens of 18 old (24–27 months), 4 middle-aged (9-11 months) and 8 young (4–5 months) mice. Six of the old animals were aged in our laboratory; all others were supplied at the specified age by Charles River.

RESULTS

Mixed lymphocyte reaction

Spleen cells from individual mice of 2 different age groups were co-cultured to see whether there is a tendency for syngeneic mixed lymphocyte reaction indicating the appearance of new or altered antigens on lymphocytes with age. The different combinations and a representation of responses obtained with 3 young and 3 old mice is shown (Table 1). The level of stimulation is measured by the ^{3}H-thymidine uptake in relation to the uptake seen when the responder and stimulator cells are from the same mouse. The amount of uptake (CPM/culture) is not very high, indicating that the MLRs were usually weak.

Summary of the one-way MLRs of 18 young and 18 old mice is given (Table 2). There were no statistically significant differences among the 4 stimulator-responder groups ($P = 0.38$). No differences dependent on the age-range of the young (2–6 months) or old (24–31 months) mice were observed. The highest percentage of responses was in the old → old group; the lowest was in the young → young.

TABLE 1. REPRESENTATIVE MIXED LYMPHOCYTE REACTION BETWEEN 3 YOUNG AND 3 OLD MICE

Source of Responder Cells	Source of Stimulator Cells					
	Young Mice			Old Mice		
Young Mice	1	2	3	1	2	3
1	--	0	0	0	0	0
2	1193	--	807	0	0	0
3	281	0	–	0	0	0
Old Mice						
1	8	758	1046	–	0	2152*
2	445	567	1612*	283	–	1921*
3	1022	217	586	262	468	–

Values given are CPM above controls (−). *Statistically significant responses ($P < 0.05$) are marked with an asterisk. There were no significant young → young or young → old responses in this particular group.

TABLE 2. SUMMARY OF ONE-WAY MIXED LYMPHOCYTE REACTIONS BETWEEN YOUNG AND OLD MICE

Responder → Stimulator	Number of Combinations[a]	Number with Significant Responses	Mean Significant Response (CPM above Control)
young → young	39	3 (7.7)[b]	2469
young → old	52	7 (13.5)	1177
old → young	55	10 (18.2)	2238
old → old	38	9 (23.7)	1492

[a] Each combination consists of the response of one mouse to cells from another mouse (e.g., young #2 → young #3). Values shown are number of combinations in that responder-stimulator group, summed over the 18 young and 18 old mice.

[b] Percentage of number of combinations.

Ben-Sasson *et al.* (1977) and Callard *et al.* (1979) have shown that polyethylene glycol (PEG) can enhance a weak MLR. With the addition of PEG to the cultures of 4 young and 4 old mice, the percentage of responses increased for young → young, young → old, old → old, but not for old → young (Table 3). The percentage increase in old → old is significant compared to the other groups. However, the net CPM above control is greatly enhanced in the young → old group (10,980 CPM). We also observed the effect of PEG on the MLR between 2 young and 2 old mice as compared to a MLR without PEG using the same animals. The presence of PEG generally increased the number of responses as compared to the same animals without PEG (Table 4). Whereas 2 of 12 combinations for all groups combined showed responses without PEG, 6 of 12 responded in the presence of PEG.

Cytotoxicity of young anti-old serum

Detection of new determinants by complement-dependent antibody-mediated cytotoxicity was used as a second approach. Young C57BL/6 mice were immunized with spleen and lymph node cells from old C57BL/6 mice to raise antibodies against the antigens of lymphoid cells from old mice (young anti-old serum). There was no cytotoxicity toward any of the young or middle-aged mice. C57BL/6 mice aged in our university facility and at Charles River Breeding Laboratories were used along with middle-

TABLE 3. MIXED LYMPHOCYTE REACTION BETWEEN YOUNG AND OLD MOUSE SPLEEN CELLS IN THE PRESENCE OF POLYETHYLENE GLYCOL

Responder → Stimulator	Number of Combinations[a]	Number with Significant Responses	Mean Significant Response (CPM above Control)
young → young	9	2 (22.2)[b]	3409
young → old	15	3 (20.0)	10980
old → young	12	0 (0)	(0)
old → old	10	4 (40.0)	2168

[a] Each combination consists of the response of one mouse to cells from another mouse (e.g., young #2 → young #3). Values shown are number of combinations in that responder-stimulator group for the 4 young and 4 old mice.

[b] Percentage of number of combinations.

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TABLE 4. EFFECT OF POLYETHYLENE GLYCOL ON MIXED LYMPHOCYTE REACTION BETWEEN YOUNG AND OLD MICE

Responder → Stimulator	Number of Combinations	Number with Significant Responses	
		Without PEG	With PEG
young → young	2	0	1
young → old	4	0	1
old → young	4	1	3
old → old	2	1	1

aged and young mice to detect the presence of antibody against old lymphocytes. The antiserum was cytotoxic to a significant number of spleen lymphocytes from 5 of 6 old mice aged at the University of Alabama in Birmingham, but only to 2 of 12 old mice aged at Charles River (Table 5).

Cytotoxicity of sensitized young lymphocytes

Our third approach for the detection of alterations in lymphocyte membrane-associated antigens involved sensitization of lymphocytes from young mice against lymphocytes from old mice in a one-way MLR. Following 5 days of culture, the viable responder cells were harvested and analyzed for cell-mediated cytotoxicity in a ^{51}Cr-release assay of PHA-stimulated old target cells. There was essentially no release of chromium in any of these assays (Table 6), whereas sensitized effector cells from an allogeneic strain (BALB/c) showed significant cytotoxicity toward C57BL/6 target cells. The percentage of viable responder cells recovered from the MLR (100% × cell number after 5 days culture/cell number at beginning of MLR) was determined. In one case recovery of viable cells was greater than 100%, indicating significant stimulation of the lymphocytes. Even here, however, as in the other experiments, there was no cytotoxicity toward cells from old mouse.

DISCUSSION

Although our MLRs indicated some possible antigenic differences with age, there were more old → old responses than young → old or old → young. Furthermore, PEG seemed

TABLE 5. COMPLEMENT-DEPENDENT ANTIBODY CYTOTOXICITY OF YOUNG ANTI-OLD SERUM AGAINST SPLEEN CELLS FROM OLD, MIDDLE-AGED AND YOUNG MICE

Donor Mice and Location	Age (months)	Number	% of Lymphocytes Killed[a]	
			Young Anti-Old Serum	Normal Mouse Serum
Old (Alabama)	24–27	5	64 ± 4.5	13.2 ± 4.2
		1	32	30
Old (Charles River)	24–27	2	18 ± 1.5	5 ± 0
		10	13 ± 2.5	16 ± 3.6
Young	4–5	8	14 ± 3.0	11 ± 2.3
Middle-Aged	9–11	4	9 ± 2.0	13 ± 2.5

[a]Mean ± S.E.M.

TABLE 6. INDUCTION OF CYTOTOXIC LYMPHOCYTES *in vitro* AND DETERMINATION OF CYTOTOXICITY IN A ^{51}CR-RELEASE ASSAY

Experiment No.	Percentage Cells Recovered	Percentage ^{51}Cr Released[a]	
		Sensitized Cells	Control
1[b]	147	−8	−1
2[b]	42	−9	−1
3[c]	9	−5	−9
4[c]	10	−8	−9
5[c]	47	−3	−1
Trial 1[d]	36	37	−3
Trial 2[d]	36	38	−7

[a]Calculated from the formula 100% × (sample release–spontaneous release)/(maximum release–spontaneous release). Values given are for ratio of sensitized cells to target cells of 200:1.

[b]Experiments 1 and 2 had entire spleen populations.

[c]Experiments 3, 4 and 5 had adherent cells removed at the start of the MLR by incubating the culture for 3 hrs. and transferring to a new flask. Phorbol myristic acetate was added in Experiment 5.

[d]Trial experiments consisted of C57BL/6 target cells and BALB/c effector cells, using entire spleen populations in the sensitization.

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to enhance old → old and young → young responses as well as young → old. This could be interpreted as an age-related increase in the factors responsible for the syngeneic MLR (von Boehmer and Adams, 1973; Smith *et al.* 1979). These findings are more closely in accord with those of Moore *et al.* (1981), who observed responses in fewer than half of the young → old and in none of the old → young, rather than with those of Callard *et al.* (1979) who had responses in nearly all of the young → old and in none of the young → young or old → old. In both these studies animals were aged at the investigators' own facilities. We found that the source of the old animals significantly affected their antigenic properties as measured in the young vs. old cytotoxocity experiments. The results of these studies were almost always negative for cytotoxicity except when the mice were aged at the University of Alabama in Birmingham, in which case results were almost all positive. Possible explanations are that viruses or altered antigens resulting from infections or lymphocyte changes due to environmental mutagens might be present in mice from some facilities.

Our results agree with the findings of Gozes *et al.* (1978) of old → old as well as young → old immunological reaction. The studies tend to indicate that the putative antigenic changes in old animals are not uniform across any population, so that there would not be some unique age-related antigen present in all old animals. These changes may be caused by mutations in lymphoid cells during the life of the animal, which could also lead to the increased incidence of different degrees of autoimmunity.

Studies on antigenic changes during *in vitro* aging of cells have been inconclusive. Miggiano *et al.* (1970) and Brautbar *et al.* (1972) reported that HLA antigens remained stable on human fibroblasts throughout their *in vitro* lifespan. Others (Sasportes *et al.*, 1971; Goldstein and Singal, 1972; Aizawa *et al*, 1980) have found evidence for a loss or alteration of antigen(s) during aging *in vitro*.

Our results do not show any ubiquitous antigenic differences between lymphocytes from young and old mice. We have shown that antigenic changes sometimes arise with

age, but it is questionable whether they can be considered a true manifestation of aging, since they are not universal in a population.

SUMMARY

Antigenic differences between lymphocytes from young and old female C57BL/6 mice were studied using 3 techniques. In one-way mixed lymphocyte reactions significant responses were obtained in 7.7% of the young → young combinations, 13.5% of the young → old, 18.2% of the old → young and 23.7% of the old → old. Polyethylene glycol enhanced the weak syngeneic MLR responses in all groups. Young anti-old antiserum was raised in young animals by injecting with lymphoid cells from old mice. The serum was cytotoxic to spleen cells from 5 of 6 old mice aged in animal facilities at University of Alabama in Birmingham and from 2 of 12 mice aged at Charles River. Lymphocytes from young mice were sensitized to old lymphocytes in a mixed lymphocyte culture and their cytotoxicity toward old cells measured in a chromium-release assay. No cytotoxicity was observed with sensitized lymphocytes from 5 young mice under various conditions. These results do not support the hypothesis that altered antigenic determinants are always or even usually present on the lymphocytes of old mice.

Acknowledgements—We thank Gretchen Cloud for her expert assistance with the statistical analysis. This work was supported in part by the National Institute on Aging training grant T32 AG00042 and research grants AG02327 and 128M.

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Experimental Gerontology, Vol. 17, pp. 383–385, 1982
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0531-5565/82/050383-03$03.00/0
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THE RELATIONSHIP OF THE GOMPERTZ CONSTANT AND MAXIMUM POTENTIAL LIFESPAN TO BODY MASS

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(*Received* 23 *November* 1981)

Abstract—The annual increase in mortality rate of eutherian mammals (α in the Gompertz equation) scales as:

$$\alpha = 0.71\ M^{-0.27 \pm 0.041}$$

Consequently, the mortality rate doubles in a time (t_d, yrs):

$$t_d = 0.693\alpha^{-1} = 0.98\ M^{0.27}$$

This scaling is very similar to that for psysiological time scales in general, $M^{1/4}$, suggesting that the ageing process may be an integral part of the physiological lifespan.

DISCUSSION

THE PRODUCT of maximum potential lifespan and the Gompertz α (herein cited), characterizing the age-dependence of mortality rate, has been shown to be almost constant over a wide range in animal sizes by Rosen *et al.* (1981). The identification of such a pattern in life-history characteristics offers encouragement for those who seek to understand the mechanisms of life processes such as aging.

The surviving fraction (n) of a population in a Gompertz decay curve is:

$$n = n_o \exp \frac{R_o}{\alpha} [1\text{-}\exp^{\alpha t}]$$

when n_o = initial population; R_o = initial mortality rate; α = Gompertz "constant" for increase in mortality rate with t = time. From a list of 23 species of animals, calculated values range from 2.2 to 12.8 for the product $\alpha\ t_{ls}$, where t_{ls} is the maximum lifespan observed, usually in the protected environment of captivity, taken as an index of maximum potential lifespan (T, in Rosen *et al.* 1981).

Those authors derived a relationship between lifespan (t_{ls}) and mass-specific metabolic rate ($\dot{H}_m/M$) from equations of Sacher (1959):

$$t_{ls} \propto \frac{1}{(\dot{H}_m/M)^{3/4}}$$

where M is body mass in kg (I have substituted different symbols to conform to standard usage for the following allometric analysis). Stahl (1962) concluded that this lifespan relationship was more directly to $\dot{H}_m/M$ rather than $(\dot{H}_m/M)^{3/4}$, the product being an essentially size-independent constant (Stahl, 1962):

$$\frac{70.5\ M^{0.73} \times 365 \times 7.52\ M^{0.29}}{1.0\ M^{1.0}} = 1.9 \times 10^5\ \text{kcal/kg} = 7.9 \times 10^5\ \text{kJ/kg}$$

A similar relationship has been observed in birds (Lindstedt and Calder, 1976, 1981).

Another approach is via regression analyses of the mammalian data in Table 1 of Rosen *et al.* (1981), although the sample size is small. Body mass was not given in that table, but mass data for these species from other sources can be used, the allometric analysis over a size range of ~ 23 g to over 400 kg being robust in the face of even 50% errors in body mass at a given size.

Such analyses yield the following relationships:

$$\alpha = 1.1\ M^{-0.36};\ r^2 = 0.63$$

$$t_{ls} = 6.9\ M^{0.28};\ r^2 = 0.77$$

From these data, their product, $\alpha\ t_{ls}$, can be obtained by allometric cancellation, empirically:

$$\alpha\ t_{ls} \propto M^{-0.36} \times M^{0.28} \propto M^{-0.08}$$

This residual mass index is probably not significantly different from a size-independent constant ($\propto M^0$). This is reinforced by regression analysis of their $\alpha\ t_{ls}$ values for these mammals:

$$\alpha\ t_{ls} = 5.8\ M^{-0.01};\ r^2 = 0.009$$

In fact, when two values are corrected in Rosen *et al.*'s table (*Microtus (arvalis) orcadensis*: $\alpha = 0.024\ W^{-1}$ vs. 0.24; *Mus musculus*: $\alpha = 0.06\ W^{-1}$ vs. 0.63) and ten additional data points are added from Sacher (1978; converting according to $T_d = 0.693\ \alpha^{-1}$):

$$\alpha = 0.71\ M^{-0.266};\ r^2 = 0.6219$$

The standard error of exponent is ± 0.04, which extends across most of the range in exponents previously given for maximum mammalian t_{ls}, 0.20 to 0.29.

Thus, direct allometric analysis seems to strengthen the case for the conclusion of Rosen *et al.* that $\alpha\ t_{ls}$ is a design constant for the description of mammalian longevity. Either of the factors, α and t_{ls}, can yield reasonably tight correlations with a host of other size-dependent variables. Although we must continually remind ourselves that correlation does not necessarily establish causation (Calder, 1976), some such pattern should eventually tell us a great deal, providing fuel for the imagination in the interim.

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The expression for the annual increase in mortality rate (α) can be reconverted to Sacher's (1978) mortality rate doubling time (yr.) as:

$$t_d = 0.693\alpha^{-1} = 0.693\,(0.71\,M^{-0.266})^{-1} = 0.98\,M^{0.266}$$

Lindstedt and Calder (1981) showed a similarity in the scaling of physiological time scales to body mass for 16 mammalian biological periods, including physiological homeostasis, reproduction, growth, and longevity. These functions had a mean body mass exponent of 0.25 ± 0.05 s.d. The fact that t_d is scaled in this same quantitative fashion suggests that the process of aging may be a fundamental part of the continuum of physiological time. The relationships between life expectancy calculated from the foregoing equations and annual natality will be discussed in a forthcoming paper (Calder, MS).

SUMMARY

Allometric analyses of the Gompertz "constant" α and maximum lifespan support the conclusion of Rosen *et al.* that the product of these aging characteristics is essentially a design constant. The body-mass dependencies of these characteristics is refined and found to conform to Stahl's scaling principles.

Acknowledgements—Supported in part by NSF grant DEB 79-03689.

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Experimental Gerontology, Vol. 17, pp. 387–397, 1982
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OL.
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SECRETION OF α-AMYLASE FROM PAROTID LOBULES OF YOUNG AND OLD RATS

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Abstract—The rate of release of a secretory enzyme, α-amylase, from the parotid lobules of 2 and 24 month old rats has been compared to determine whether the secretory activity of the cells change during aging. Upon incubation in the presence of a secretogogue, isoproterenol (10^{-5} M), about the same proportions of the glandular α-amylase are released at about the same rate from these lobules. The isoproterenol-stimulated release of the enzyme is inhibited nearly completely by preincubating the lobules with propranolol (10^{-5} M) in both age groups, indicating that the enzyme secretion occurs through the stimulation of β-receptors. When viewed in the electron microscope, the cell membranes bordering the secretory lumen in isoproterenol-incubated lobules reveal festooned appearances which suggest that the enzyme release occurs by means of exocytosis. These observations indicate that the ability of the parotid cells to release secretory products through the β-receptor mediation does not significantly change with increasing age.

INTRODUCTION

SOME ASPECTS of the salivary gland function in humans undergo changes with increasing age. The rate of basal salivary flow, as well as the stimulated secretion of saliva, decreases with age (Bertram, 1967; Meyer and Necheles, 1940). Also, the content of amylase in mixed saliva is reduced in elderly persons (Chilla, *et al.*, 1974; Meyer and Necheles, 1940; Meyer, *et al.* 1937), indicating the reduction in the secretory protein content of the saliva.

These reductions in the salivary flow and the protein content suggest that the processes of synthesis and/or release of secretory proteins decline with age. In our previous studies with parotid glands of the rat, we have demonstrated that the cellular level of protein synthesis progressively declines with increasing age as determined by the reduction in the rate of incorporation of radioactive amino acids into the acid-insoluble proteins (Kim, 1981, Kim, *et al.*, 1980; 1981) or secretory proteins (Kim, *et al.*, 1982) of the gland. Furthermore, this decline seems to reflect specific changes in the synthesis of proteins in this gland, and not changes in the cellular metabolic activity with aging (Kim, *et al.*, 1980).

Previous studies have shown that the processes of synthesis and release of secretory proteins are interrelated in exocrine secretory cells. In pancrease and parotid glands of rats,

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the rate of protein synthesis increases following the stimulated secretion of glandular content (Grand and Gross, 1969; Lillie and Han, 1973; Sreebny, *et al.*, 1971). Then, the age-related reduction in the rate of protein synthesis in parotid glands of rats might be related to the decline in the secretory activity of the glandular cells.

The secretory activity of the salivary gland is controlled largely by the autonomic nervous system (Schneyer and Hall, 1967), and the release of secretory proteins is induced by epinephrine (Bdolah, *et al.*, 1964; Bdolah and Schramm, 1965). The release of amylase from parotid glands of the rat is strongly stimulated by agents with β-agonist activity such as isoproterenol (IPR) and the catecholamines (Bdolah, *et al.*, 1964; Nelson and Stouffer, 1972; Butcher *et al.*, 1975). Following a single injection of IPR, a near complete discharge of α-amylase from parotid glands of the rat takes place within 2 hours (Malamud, 1972; Lillie and Han, 1973).

The release of α-amylase can be induced also by incubating parotid gland slices or lobules in the presence of IPR (Castle, *et al.*, 1972; Kusek, 1979). The process of amylase secretion through β-receptor activation seems to involve cyclic AMP (Babad, *et al.*, 1967; Bdolah and Schramm, 1965; Butcher and Goldman, 1974), and can be prevented with β-adrenergic blocker propranolol (Castle, *et al.*, 1972; Kusek, 1979; Malamud, 1972).

This study was undertaken to determine whether there are age-related changes which affect the ability of parotid gland cells to release secretory proteins. This was done by comparing the cellular level of α-amylase and profile of release of the enzyme from parotid lobules of 2 and 24 month old rats upon incubation with IPR. In order to determine whether the IPR-stimulated release of α-amylase in the older rats also occurs by means of exocytosis as in young rats (Amsterdam, *et al.*, 1909), morphological observations of the lobules were made at various times during the incubation. In addition, some lobules were preincubated in the presence of propranolol to examine whether the IPR-stimulated release of the enzyme is inhibited by a β-blocker.

MATERIALS AND METHODS

Animals

Male, Sprague-Dawley rats of 2 and 24 months of age were used and obtained from Charles River Breeding Laboratories in Portage, Michigan. The 24 month old rats came from the colony of retired breeders which were grouped into cohorts between 10 and 12 months of age. Therefore, the rats in this age group ranged from 23 to 25 months. All rats were maintained behind a barrier containment facility and were screened regularly for known pathogens. These rats are also free of immunological titers for sialodacryoadenitis (SDA) virus which affects the submandibular and parotid glands of rats and is widespread in many commercial stocks of rats. Rats were fed ad libitum and were housed under controlled light and dark conditions for more than three days before using them for experiments. Food was withheld for about 16 hours before beginning an experiment, but rats were allowed access to drinking water. Rats were killed by cervical dislocation.

IRP-stimulated release of α-amylase

After killing the rats, the parotid glands were removed and immersed in Eagle's Minimum Essential Medium (MEM) that had been warmed to 37°C (Babad *et al.*, 1967). The medium was gassed with a mixture of 95 percent O_2 and 5 percent CO_2. The removed glands were cleaned of connective tissue, blood vessels, nerves and large ducts in the central region. Parotid lobules were isolated manually with Dumont #5 forceps with fine tips under a dissecting microscope. During the lobule separation, the gas mixture was continuously bubbled into the medium through a thin glass tubing. Lobules from at least 2 rats were pooled and preincubated in 15 ml of gassed MEM at 37°C for 15 minutes to wash out amylase from the ducts or the damaged cells. The lobules were placed in a 50 ml flask containing 10 ml of MEM supplemented with desired concentrations of IPR (dl-isoproterenol, City Chemical, New York) and incubated at 37°C in a water bath which was continuously agitated at 80 cycles/min. During incubation, 50 μl of fresh IPR solution was added to each flask every 30 minutes to replace used up and oxidized IPR (Castle *et al.*, 1972).

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At various time periods, 1 ml of medium was withdrawn for amylase assay and replaced with 1 ml of fresh MEM. A small amount of IPR solution was also added to the medium to make up for the secretogogue depleted by medium removal. During this experiment, a fresh IPR stock solution was prepared every hour to eliminate errors in the drug concentration due to oxidation. For controls, the lobules were incubated in MEM with an equal volume of distilled water replacing IPR.

To determine the effect of a β-blocker on the IPR-stimulated release of α-amylase, the lobules were preincubated for 30 min. in the medium containing dl-propranolol hydrochloride (Ayerst, New York). The control lobules were incubated in the medium with the same amount of distilled water replacing propranolol. Following preincubation, the lobules were further incubated in MEM supplemented with 10^{-5} M IPR. The incubation medium also contained the same concentration of propranolol as in the preincubation medium. The control lobules were divided into 2 groups following preincubation with propranolol. One group was incubated in MEM supplemented with 10^{-5} M of IPR and the other group in MEM containing an equal volume of distilled water. At the end of various time intervals, 1 ml of medium was withdrawn for amylase assay. The medium was replaced each time, and a small amount of IPR and propranolol was added to the medium to bring up the concentrations to the original levels.

Assays of Amylase Release

The assay of α-amylase activity was done by using the Roche Amylochrome assay kit (Roche Diagnostics, Nutley, N.J.) as described previously (Kim, 1981). This is a colorimetric procedure of measuring the amount of dye released from the Cibachron Blue F3GA-amylose complex upon hydrolysis of the α1–4 bonds of the amylose by the action of α-amylase.

Tissue amylase was extracted by an extensive homogenization of lobules in phosphate buffered saline (PBS). An aliquot was removed from the homogenate, centrifuged at 25,000 xg for 10 minutes and the supernatant was used for α-amylase activity determination. The medium samples collected at various times during incubation and the tissue samples at the end of incubation were assayed for α-amylase activity.

The cumulative amount of α-amylase activity released into the incubation medium was followed with time. The cumulative amount in the medium and the amount of activity remaining in the tissue at the end of incubation constituted the total enzyme activity. Percentage release of α-amylase was calculated from the enzyme activity released into the medium and the total activity. The DNA contents of the tissue were determined according to Burton (1958).

Morphological Observations

The glandular lobules were prepared for morphological observations at various time periods during the incubation. These lobules were fixed in the fixative containing paraformaldehyde and glutaraldehyde, post-fixed in osmium and processed for viewing in the light and electron microscope as described previously (Kim, 1981; Kim, *et al.*, 1980). Thick sections (1 μm), after staining with toluidine blue, were used for light microscopy. The blocks were further trimmed and thin sections (about 700 A) were cut for viewing in a Hitachi 11c electron microscope.

RESULTS

The α-amylase content in parotid glands

The cellular contents of α-amylase in parotid glands in these rats do not appear to change between the ages of 2 and 24 months. The level of α-amylase activity in the glands, compared based on the DNA content, is not significantly different at these ages (Table 1). However, the DNA content/mg tissue of the gland is significantly different at these two ages (Table 1), and this might reflect the age-related increase in the amount of glandular tissue contents.

The IPR stimulated release of α-amylase

The lobules prepared from the glands of both age groups release α-amylase spontaneously at a fairly stable rate for up to 4 hours of incubation in MEM, at 37°C (unpublished observation). The amylase release is greatly stimulated upon addition of IPR in the medium, and this stimulated release is dependent upon the concentrations of the

TABLE 1. LEVELS OF α-AMYLASE ACTIVITY AND CONTENTS OF DNA IN PAROTID GLANDS

Age Mos.	No. of Rats	No. of Samples	D.U.[a] / MG Tissue ± S.E. × 10^3	D.U.[a] / MG DNA ± S.E. × 10^6	μG DNA / MG Tissue ± S.E.
2	4	16	152.2 ± 7.8	40.3 ± 2.8[b]	3.86 ± 0.09[c]
24	3	14	151.0 ± 8.9	47.7 ± 3.0[b]	3.19 ± 0.08[c]

[a] Dye Units – 1,050 D.U. = 1 unit of α-amylase activity which produces 1 mg of maltose in 3 minutes at 37°C.
[b] Not significantly different ($0.05 < P < 0.1$)
[c] Significantly different ($P < 0.001$)

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secretogogue (Fig. 1). The maximum release of α-amylase is obtained with 10^{-5} M IPR in the medium, and, therefore, this concentration was used throughout the study in comparing the stimulated release of α-amylase.

There is no significant difference in the IPR-stimulated release of α-amylase by the lobules from 2 and 24 month old rats (Fig. 2). The lobules from the glands of these rats of both ages release about the same proportions of the total amylase at about the same rate

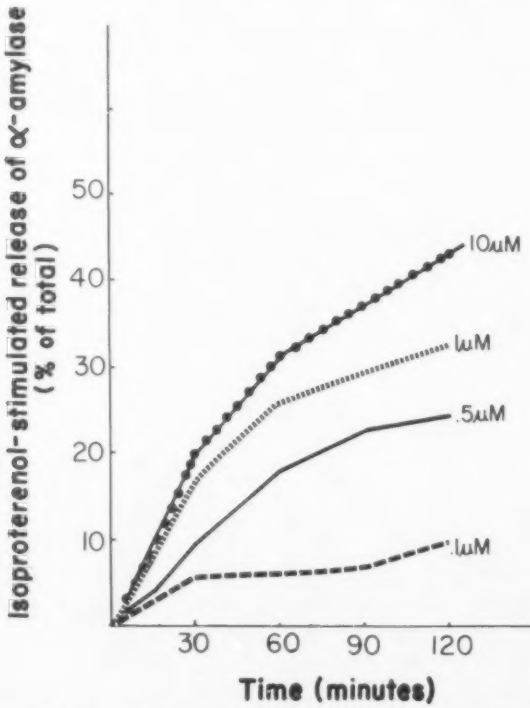


FIG. 1. Dependence of α-amylase release from parotid lobules on the concentration of isoproterenol. Parotid lobules from 2-month-old rats were incubated in the presence of varying concentrations of the secretogogue. The percentage of α-amylase released into the medium was calculated for this figure, as well as the other ones, as described in the Methods.

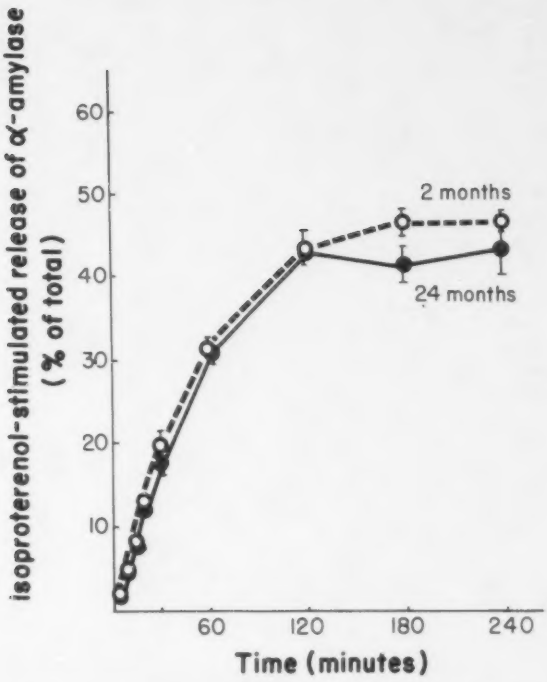


FIG. 2. Comparisons of the release profile of α-amylase from the parotid lobules of 2 and 24 months old rats upon incubation with isoproterenol in the concentration of 10^{-5}M in the medium. Values represent means ± S.D. of 3 experiments.

upon incubation with IPR (Fig. 2). The release of amylase seems to occur in 2 phases. The rate of release increases rapidly in the initial phase of incubation with IPR, reaching the highest rate at 20 minutes in both groups (Figs. 3a and 3b). After this phase, the rate of release declines gradually and this decline is paralleled in both age groups.

The effects of β-blocker on the IPR-stimulated release of α-amylase

The release of α-amylase is inhibited by propranolol in both age groups when the lobules are preincubated with the β-blocker before stimulating with IPR (Fig. 4). Following preincubation of the lobules in the presence of 10^{-5} M propranolol, the IPR-stimulated release of α-amylase is initially blocked almost completely in both groups. The inhibitory effect of propranolol decreases with time. However, the amylase release at the end of a 1 hour incubation with IPR is still inhibited by about 90 percent of the level released by the control lobules in both age groups.

Morphological appearances of the incubated lobules

The morphological changes which occur in the lobules during the incubation with IPR are similar in cases of the young and old rats. As illustrated with the lobules of 24 month old rats in Figs. 5–8, the acini become devoid of secretory granules within 30 minutes of

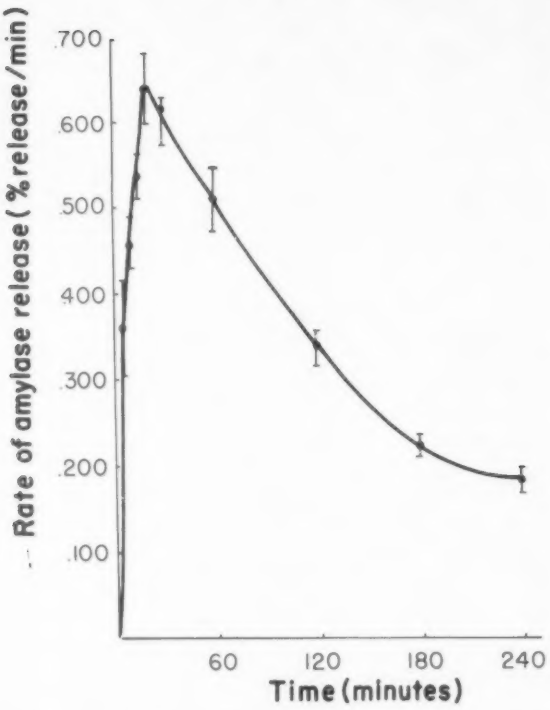


FIG. 3a. The rate of release of α-amylase at various time periods during incubation of the lobules from 2 month old rats in the presence of isoproterenol (10^{-5}M). Values represent means ± S.D. of 4–8 samples.

incubation. In the lobules incubated for 10 minutes with the secretogogue, the numbers of the secretory granules are reduced while the lumens of the acini are widened (Fig. 5). These changes are more pronounced at 20 minutes (Fig. 6). At the end of 30 minute incubation (Fig. 7), secretory granules are absent in most of the acinar cells. On the other hand, in the lobules incubated for 30 minutes in the medium without IPR (Fig. 8), the cells retain secretory granules and these lobules are similar in appearance to the unincubated lobules (not shown). When viewed in the electron microscope (Fig. 9), the widened lumens are bound by the acinar cell membranes which protrude deep into the cytoplasm in many places. The luminal space reveals amorphous substances.

DISCUSSION

The results of our *in vitro* release experiments in this study indicate that the ability of the parotid gland cells to secrete proteins upon stimulation by secretogogues does not change with age. This is evidenced by the similarities shown by the lobules of 2 and 24 month old rats in releasing α-amylase upon incubation with IPR. The isolated lobules of these two age rats release about the same proportion of tissue amylase at about the same rate upon incubation with the secretogogue. Similar results have been reported previously

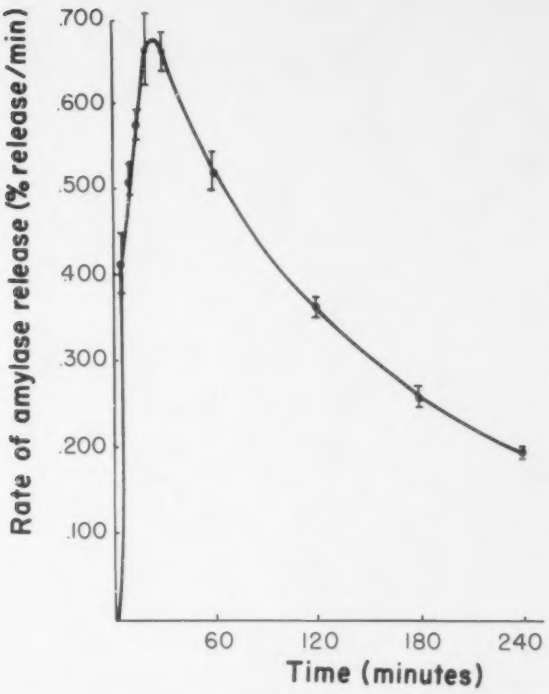


FIG. 3b. The rate of release of α-amylase at various time periods upon incubation of the lobules from 24 month old rats in the presence of isoproterenol (10^{-5}M). Values represent means ± S.D. of 4–8 samples.

by others using enzymatically dispersed cell aggregates from rat parotid glands of varying ages (Ito, *et al.*, 1981).

The IPR-stimulated release of amylase, observed in this study, is mediated by β-adrenergic receptors and occurs by the process of exocytosis as demonstrated by the study with propranolol and morphological observations of the incubated lobules, respectively. The IPR-stimulated release of amylase is inhibited by preincubating the lobules in the presence of this β-blocking agent and the effect of it reduces with time in both age groups. It has been shown that propranolol binds exclusively to the β-receptors (Levitzki, *et al.*, 1974) and that the bound propranolol becomes displaced by IPR with time in a concentration dependent manner (Levitzki, *et al.*, 1974; Atlas, *et al.*, 1974).

The discharge of secretory granule contents occurs by the fusion of the granule membranes with the cell membranes at the luminal surface as shown morphologically in parotid glands of young rats (Amsterdam, *et al.*, 1969; Lillie and Han, 1973). This is the case also in the glands of older rats as demonstrated in this study. During the incubation with IPR, the number of secretory granules decreases as the lumens widen in the parotid lobules. The festooned appearance of the lumen in the incubated lobules probably resulted from the fusion of the granules with the cell membrane during exocytosis. Thus, the IPR-stimulated release of α-amylase from the lobules of young and old rats represents the true secretory activity of the gland and not the leakage of the enzyme during incubation.

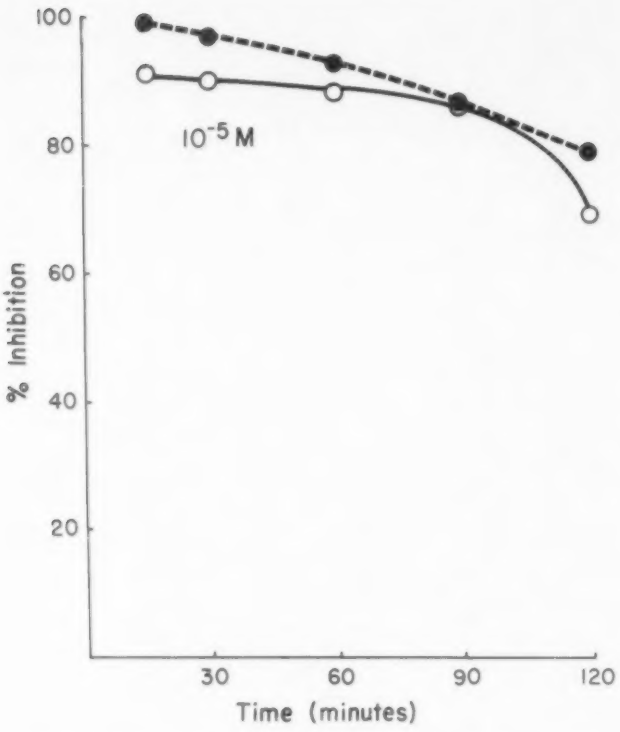
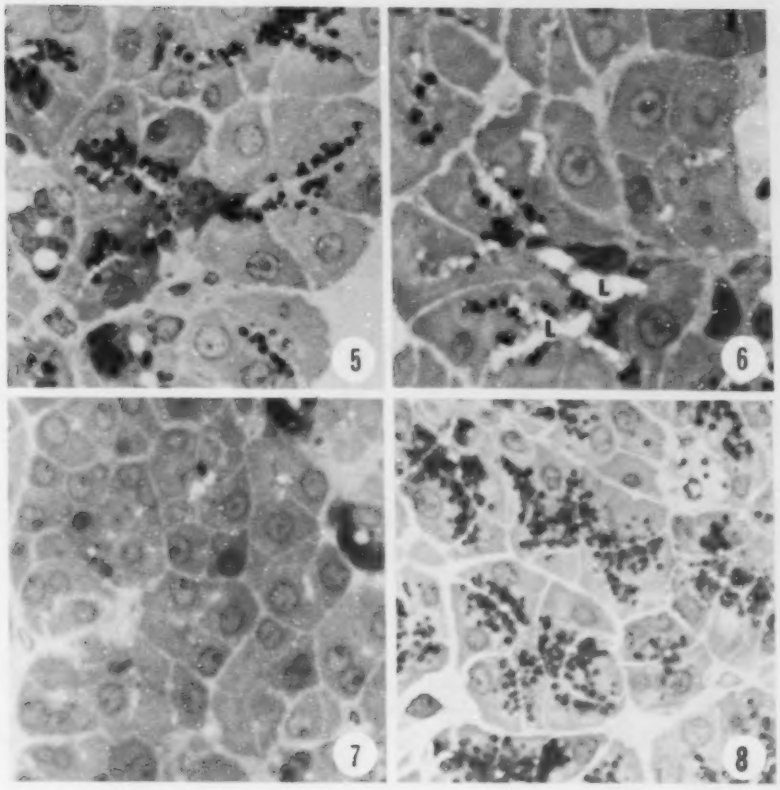


FIG. 4. The effect of a β-adrenergic blocker on the isoproterenol-stimulated release of amylase from the lobules of 2(-•-) and 24(-o-) month old rats. The secretogogue-stimulated release of amylase is inhibited nearly completely by preincubating with propranolol (10^{-5}M) in both groups.

Then, it appears that the age-related decline in protein synthesis in this gland (Kim, *et al.*, 1980; 1981; 1982; Kim, 1981) occurs unrelated to the reduction in the ability of the secretory cells to release proteins. It is possible, however, that the reduced level of protein synthesis in old rats is related to the general decline in the secretory activity of the gland, presumably due to the reduced level of digestive activity with age. Thus, the secretory proteins which are stored in cells for prolonged periods of time might have an inhibitory effect on the synthesis of more proteins.

In fact, there is no significant difference in the cellular level of α-amylase activity in the glands of 2 and 24 month old rats, despite the reduction in the level of protein synthesis in the older rats. Since this enzyme makes up a large proportion of the secretory protein produced by this gland (Schramm, 1968), the results indicate that the cellular level of secretory proteins does not change with age. This is the case regardless of whether the comparison is made based on the wet weight or the DNA content of the gland. The reduction in the amount of DNA per unit weight of the gland in the older rats probably reflects the increase in the amount of nonsecretory tissues, such as fat, fibrous connective tissue and others during aging. The age-related increase in the amount of fat and other supportive tissues



FIGS. 5–8. Light micrographs showing parotid lobules from 24 month old rats incubated in the medium containing 10^{-5} MIPR for 10 (Fig. 5), 20 (Fig. 6) and 30 (Fig. 7) minutes. The lobule shown in Fig. 8 was incubated for 30 minutes in the medium without IPR as a control. With increasing time, the number of secretory granules decreases while the lumen (L) of the acini widens. After incubating for 30 minutes, the acinar cells are devoid of secretory granules. On the other hand, the lobules incubated without IPR retain most of the secretory granules even after a 30 minute incubation (×1,000).

has been reported to occur in parotid glands and other salivary glands (Andrew, 1949; Bogart, 1970).

Previously, we have reported that the cellular level of α-amylase declines with age in parotid glands of rats (Kim, 1981). However, these rats, obtained from the aging colony maintained at Charles River Breeding Laboratories in Wilmington, Massachusetts, showed positive titers for sialodacryoadenitis viruses. Therefore, the reduced level of amylase in the gland of these rats could have reflected the clinical manifestations of this salivary gland disease.

SUMMARY

In order to determine whether there are changes in the cellular secretory activity during aging, the ability of parotid acinar cells to release α-amylase has been examined. This was done by comparing the release profiles of the enzyme from the glandular lobules of 2 and

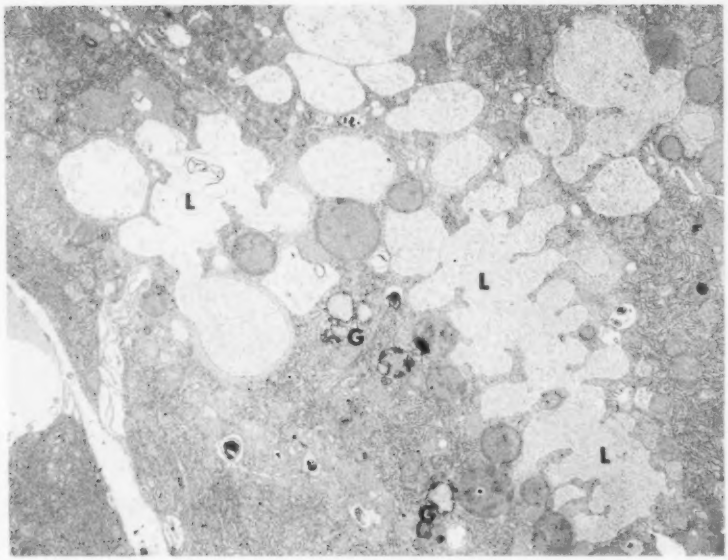


FIG. 9. Electron micrograph of a lobule incubated for 20 minutes in the presence of IPR (10^{-5}M) showing widened lumens (L) of the acinus. The festooned appearance of the lumen probably resulted from the fusion of the secretory granules with the cell membrane. The amorphous substance present in the luminal space is likely to be the secreted contents of the granules. Lipofusion granules (G) which are present commonly in the glands of old rats are indicated (×7,000).

24 month old rats upon stimulation by incubating in the presence of a secretogogue, isoproterenol (IPR). The lobules from these two age rats release about the same proportions of the tissue α-amylase at about the same rate upon incubation with IPR (10^{-5} M). The IPR-stimulated release of the enzyme is inhibited similarly in these age groups by preincubating the lobules with a β-blocker, propranolol (10^{-5} M). Furthermore, the release of the secretory products from the glands of the older rats seems to occur by means of exocytosis, as is the case in young rats. These results indicate that the ability of the cells to release secretory products through the stimulation of β-receptors remains unchanged during aging in parotid glands of the rat.

Acknowledgements—Authors thank Virginia Hartog for assistance in the experiments. This work was supported by the Veterans Administration and by Grant AG 00944 from the National Institute on Aging.

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Experimental Gerontology, Vol. 17, pp. 399–405, 1982
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CHANGES IN THE CHARACTERISTICS OF COLLAGEN OF YOUNG MALE GARDEN LIZARDS FOLLOWING CENTROPHENOXINE AND LYSINE TREATMENT

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(*Received* 2 *February* 1982)

Abstract – Both centrophenoxine and lysine appear to stimulate the deposition of collagen as evident from the values of total content in skin and tendon. The solubilities of collagen in salt and acid media and the soluble/insoluble collagen ratio increased in almost all the three tissues of treated lizards though differing to some extent in the degree of response. It is suggested that both the chemicals possibly prevent cross-link formation thus retarding the aging of collagen in lizard tissues.

INTRODUCTION

VARIOUS FACTORS are known to influence the characteristics of collagen (Timiras, 1972) of animal tissues. An increase in the stability of collagen may occur due to some such factors and a decrease due to others. These alterations are the reflections of either an increase or a decrease in the number of intermolecular cross-linkages in collagen molecule. The degree of cross-link formation in collagen affects the physiological functions of the concerned tissue (Kohn, 1978). It is also likely that factors influencing the characteristics of collagen may affect other physiological indices as well (Comfort, 1974). A survey and an evaluation of some such factors, therefore, seems to be appropriate.

Earlier we have shown that some hormones (Brahma *et al.*, 1980), food restriction and varied diets (Brahma and Patnaik, 1981) alter the degree of cross-link formation in tissue collagen of young male garden lizards. The present study summarizes the results of the actions of centrophenoxine and lysine; both chemicals have been reported to affect aging rate. Clinical trials on geriatric patients have shown that centrophenoxine markedly improved the symptoms of senility such as confusion, psychosomatic asthenia and disturbances of memory (Destrem, 1961; Houillon and Salles, 1963). The drug also reduced the accumulation of lipofuscin, the age-pigment, in nervous tissue (Nandy, 1968). Lysine is an essential amino acid required for growth and maintenance of nitrogen equilibrium (Oser, 1965). We thought it worthwhile to know how far these two chemicals could modify the stability of collagen in young garden lizards.

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MATERIALS AND METHODS

Animals

The animal models used in the present study were the male garden lizards. They have a life-span of 4 years in the wild and were same as described earlier (Kara & Patnaik, 1980). The stability of collagen has been shown to increase with advancing age of these garden lizards (Panigrahy and Patnaik, 1976).

Male lizards (*Calotes versicolor*) aged below one year (Patnaik and Behera, 1981) and having an average body-weight of 9.5g and snout-to-vent (S-V) length of 73mm were collected during October 1980 from Berhampur localities (being about 10 K M away from the sea, the winter in this area is mild and summer not-so-severe) and used for the present study. After collection, the lizards were maintained in the laboratory at room temperature (28° ± 2°C) for a week. They were fed with goat liver on alternate days and tap water was provided *ad libitum*.

Experimental design

Two sets of experiments were conducted. In the first set, the experimental lizards were injected on alternate days with centrophenoxine (Helfergin, Promonta Laboratories, Hamburg) intramuscularly (0.8 mg/10g. body-weight) for three weeks; whereas, the control group of lizards received an equal volume of the vehicle (distilled water, 0.2 ml) each time. In the second set the control group of lizards were injected with an equal volume of vehicle (distilled water, 0.2 ml); whereas the experimental group received *L*-lysine monohydrochloride (BDH, England) on alternate days intraperitoneally (10 μg/10g. body-weight) for a period of three weeks. The body-weight and S-V length of both the groups of lizards belonging to two sets were measured before killing on the 22nd day by a blow on the head.

Evaluation of collagen characteristics

Weighed quantities of long bones, dorsal skin and tendons from tail and phallanges were collected from control and treated animals. Neutral salt soluble collagen fraction was extracted in 2.5 ml of 0.15 M sodium chloride (24 h at 8°C). The salt soluble and salt insoluble fractions were separated by centrifugation at 1500 rpm for 15 minutes in a Remi centrifuge (Indian make). From the salt insoluble fraction the acetic acid soluble collagen fraction was extracted in 3.5 ml of 0.45 M acetic acid (24 h at 8°C). The acid soluble and insoluble fractions were separated by centrifugation at 1500 rpm for 15 minutes; but in case of tendon, fractionation of samples was done by centrifugation at 7,000 rpm for 15 minutes in a K-24 refrigerated MLW centrifuge (German make).

Salt soluble, acid soluble and insoluble fractions were hydrolysed in 6 N HCl in sealed tubes for 16 h at 110°C. The hydroxyproline content was estimated in neutralized (with 5N NaOH and methyl red as indicator) and diluted samples by colorimetric (Erma Japan model AEII) method of Neuman and Logan (1950) as modified by Leach (1960). For converting hydroxyproline values to collagen content a multiplication factor of 7.46 was used.

RESULTS

Morphometric parameters

These data were not analysed statistically but were expressed on the basis of percentage change in average values as individual cases showed an inconsistent pattern of changes such as either a decrease or an increase in the above parameters. Such an evaluation revealed that neither the body-weight nor the S-V length of male garden lizards showed a pronounced change following chemical treatments (Table 1).

TABLE 1. PERCENTAGE OF CHANGE (BASED ON AVERAGE VALUES OBTAINED FROM 9 TO 10 CASES) IN MORPHOMETRIC PARAMETERS FOLLOWING CHEMICAL TREATMENT

Chemical	Condition	% change after 3 weeks	
		Body-weight	S-V length
Centrophenoxine	Control	+ 6.1	+4.0
	Treated	+ 2.0	+2.6
Lysine	Control	+13.6	+5.7
	Treated	+18.5	+4.2

Effect of centrophenoxine

In bone a marginal increase in salt solubility of collagen and salt soluble/insoluble collagen ratio was observed following centrophenoxine treatment. While in skin, both salt soluble/insoluble ratio and the total collagen content of skin were increased, almost all the parameters of tendon collagen barring the salt solubility were found to be enhanced following centrophenoxine treatment (Table 2).

Degree of response of tissues to centrophenoxine

The degree of accumulation of total collagen content was greater in skin than in other tissues. The increase in soluble/insoluble ratio by salt extraction was more evident in skin and tendon than in bone. Only the tendon collagen showed a significant increase in acid solubility and acid soluble/acid insoluble ratio (Table 4).

Effect of lysine

Lysine did not influence the collagen characteristics of bone appreciably. A marginal rise in salt soluble/insoluble collagen ratio was the only significant result. But the amino-acid led to an increase in almost all the characteristics of skin collagen barring the salt soluble/insoluble ratio and caused a rise in acid solubility and acid soluble/insoluble collagen ratio and the total collagen content of tendon (Table 3).

Degree of response of tissues to lysine

In general the degree of response of the characteristics of collagen was greater in skin than in other tissue. The bone showed an increase only in its soluble/insoluble collagen ratio by salt extraction method. The responses of tendon in most of the characteristics of collagen were lower than those of the skin (Table 4).

TABLE 2. EFFECT OF CENTROPHENOXINE ON THE COLLAGEN CHARACTERISTICS OF BONE, SKIN AND TENDON OF YOUNG MALE GARDEN LIZARD. VALUES ARE AVERAGE ± SEM. NUMBERS IN PARENTHESES INDICATE ANIMAL NUMBER. *P* VALUES WERE OBTAINED BY STUDENTS *t* TEST. NS, not significant at 0.1 LEVEL

Tissue	Condition	Total collagen (mg/g. wet wt.)	% of Solubility		Soluble/Insoluble × 1000	
			Salt	Acid	Salt	Acid
Bone	Control	114.25 ± 7.63 (8)	1.63 ± 0.22 (7)	1.36 ± 0.11 (7)	16 ± 2 (7)	14 ± 8 (6)
	Treated	120.12 ± 4.20 (8)	2.28 ± 0.22 (8)	1.82 ± 0.30 (8)	22 ± 2 (8)	19 ± 3 (7)
	P	NS	<0.1	NS	<0.1	NS
Skin	Control	221.67 ± 10.94 (7)	1.78 ± 0.17 (8)	7.23 ± 0.63 (8)	17 ± 1 (8)	70 ± 5 (6)
	Treated	316.86 ± 22.75 (9)	2.48 ± 0.35 (9)	6.08 ± 0.54 (7)	29 ± 3 (7)	71 ± 5 (6)
	P	<0.01	NS	NS	<0.01	NS
Tendon	Control	273.57 ± 19.59 (8)	1.88 ± 0.19 (8)	34.87 ± 2.59 (9)	18 ± 1 (8)	558 ± 62 (9)
	Treated	332.85 ± 25.05 (7)	2.59 ± 1.10 (9)	41.92 ± 2.21 (9)	31 ± 4 (6)	769 ± 61 (9)
	P	<0.1	NS	<0.05	<0.01	<0.01

TABLE 3. EFFECT OF LYSINE ON THE COLLAGEN CHARACTERISTICS OF BONE, SKIN AND TENDON OF YOUNG MALE GARDEN LIZARD. VALUES ARE AVERAGE ± SEM. NUMBERS IN PARENTHESES INDICATE ANIMAL NUMBER. P VALUES WERE OBTAINED BY STUDENTS t TEST

Tissue	Condition	Total collagen (mg/g. wet wt.)	% of Solubility		Soluble/Insoluble × 1000	
			Salt	Acid	Salt	Acid
Bone	Control	121.33 ± 5.97 (9)	1.83 ± 0.14 (8)	1.61 ± 0.24 (9)	18 ± 1 (8)	14 ± 1 (6)
	Treated	134.46 ± 7.43 (10)	2.49 ± 0.45 (9)	1.50 ± 0.24 (9)	31 ± 5 (6)	11 ± 1 (7)
	P	NS	NS	NS	<0.02	NS
Skin	Control	266.68 ± 16.45 (7)	2.45 ± 0.31 (8)	10.18 ± 1.17 (5)	20 ± 2 (8)	117 ± 15 (5)
	Treated	341.90 ± 13.01 (9)	3.87 ± 0.70 (9)	22.27 ± 1.79 (6)	24 ± 1 (7)	309 ± 34 (6)
	P	<0.01	<0.1	<0.001	NS	<0.01
Tendon	Control	276.22 ± 19.98 (9)	3.16 ± 0.31 (6)	43.93 ± 1.11 (8)	32 ± 3 (6)	841 ± 44 (8)
	Treated	365.30 ± 20.88 (8)	2.91 ± 0.38 (9)	48.50 ± 1.82 (7)	33 ± 3 (7)	1021 ± 61 (7)
	P	<0.02	NS	<0.05	NS	<0.05

DISCUSSION

We started with the assumption that both centrophenoxine and lysine might promote the growth of garden lizards morphometrically and biochemically. But the morphometric parameters i.e., the body-weight and S-V length did not show appreciable changes following the chemical treatment for three weeks. On the other hand, alterations in biochemical parameters i.e., the characteristics of collagen were clearly indicated. It is likely that the biochemical parameters respond sooner to treatment than the morphometric parameters which require treatment for a longer time.

The three important characteristics of collagen i.e., total collagen, % of solubility and soluble/insoluble ratio need consideration. Total collagen content roughly indicates the balance between the amounts synthesized and degraded. Percentage of solubility and soluble/insoluble ratio are the well-known indirect measures for assessing the degree of cross-link formation in collagen (Sinex, 1964; Hall, 1976). It appears that both centrophenoxine and lysine augment collagen synthesis and simultaneously keep the collagen in younger state by preventing cross-link formation.

That centrophenoxine reduces the accumulation of the age pigment lipofuscin in neurons of guinea pigs (Nandy and Bourne, 1966; Nandy, 1968) and in neuroblastoma cells in culture (Nandy *et al.*, 1978) have been reported earlier. It also has favorable effect on the cortical cells of old female Wistar rats (Nagy *et al.*, 1979). Stimulation of metabolism of brain tissue has been observed by both *in vitro* and *in vivo* treatment of centrophenoxine (Nickel *et al.*, 1963; Herrmann and Dittmann, 1971). Beneficial effects of centrophenoxine treatment have been observed in a series of human pathological states (Schmidt and Broicher, 1970; Vojtechovsky *et al.*, 1970).

These observations suggested that centrophenoxine might retard the aging processes in animals. Cross-link formation in collagen increases with advancing age of fish (Gantayat

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TABLE 4. PERCENTAGE OF CHANGE (BASED ON AVERAGE VALUES OBTAINED FROM 5–10 CASES AS MENTIONED IN TABLES 2 AND 3) IN CHARACTERISTICS OF COLLAGEN FOLLOWING CHEMICAL TREATMENT. VALUES IN PARENTHESES REFER TO LEVEL OF SIGNIFICANCE

Chemical	Tissue	Total collagen	Solubility		Soluble/insoluble ratio	
			Salt	Acid	Salt	Acid
Centrophenoxine	Bone	+5.1(NS)	+39.5(<0.1)	+ 33.3(NS)	+37.5(<0.1)	+ 35.7(NS)
	Skin	+42.9(<0.01)	+39.7(NS)	− 15.9(NS)	+70.5(<0.01)	+ 1.4(NS)
	Tendon	+21.6(<0.1)	+38.1(NS)	+ 20.2(<0.05)	+72.2(<0.01)	+ 37.8(<0.01)
Lysine	Bone	+10.8(NS)	+35.8(NS)	− 6.9(NS)	+72.2(<0.02)	− 21.4(NS)
	Skin	+28.2(<0.01)	+57.9(<0.1)	+118.7(<0.0001)	+20.0(NS)	+164.1(<0.01)
	Tendon	+32.2(<0.02)	− 7.9(NS)	+ 10.3(<0.05)	− 3.1(NS)	+ 21.4(<0.05)

and Patnaik, 1980), lizard (Panigrahy and Patnaik, 1976) and mammals (Sinex, 1964; Hall, 1976) and this is paralleled by a decrease in solubility and soluble/insoluble ratio. Enhanced solubilities in salt and acid media and a rise in soluble/insoluble ratio in treated animals suggest that centrophenoxine possibly prevents cross-link formation in collagen of garden lizard tissues and thus retards the aging of collagen. Our findings therefore support the beneficial effects of centrophenoxine.

Both proline and lysine are incorporated in collagen molecules and later hydroxylated as hydroxyproline and hydroxylysine. The role of lysine as a general growth promoter is well-known (Lewis and de Mello, 1968; Oser, 1965). In garden lizard tissues the amino acid seems not only to promote the synthesis of collagen but also to prevent the cross-link formation thus indicating the retardation of collagen aging.

Since both centrophenoxine and lysine appear to retard the aging of collagen it is urged that their effect on other indices of aging be explored for a better understanding of the chemical control of the aging process. Before such an undertaking, it would be desirable to confirm the beneficial effect of both the chemicals by direct estimation of the cross-link contents of collagen.

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SUMMARY

The *in vivo* effects of centrophenoxine and lysine were evaluated in bone, skin and tendon collagens of young male garden lizards. Judging from the total collagen content values both chemicals appear to stimulate the deposition of collagen in skin and tendon but not in bone. The salt solubility of bone collagen, acid solubility of tendon collagen, salt soluble/insoluble ratio of skin, tendon and bone collagens and acid soluble/insoluble ratio of tendon collagen were increased upon centrophenoxine treatment. Similarly, the salt solubility of skin collagen, acid solubilities of skin and tendon collagens, salt soluble/insoluble ratio of bone collagen and acid soluble/insoluble ratio of skin and tendon collagens were found to be enhanced following lysine treatment. It is suggested that both chemicals possibly prevent cross-link formation thus retarding the aging of collagen in lizard tissues.

Acknowledgements—Thanks are due to Promonta Laboratories (Hamburg) for a gift sample of Helfergin and to the Head of the Department of Zoology for laboratory facilities. The work was supported by a grant from University Grants Commission (New Delhi).

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Experimental Gerontology, Vol. 17, pp. 407–416, 1982
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BILIARY EXCRETION OF DIGITOXIN AND ITS METABOLITES IN YOUNG AND OLD MALE WISTAR RATS

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(*Received* 15 *December* 1981)

Abstract—The biliary excretion of digitoxin (Dt3) and its metabolites were compared between young (3-month-old) and old (25-month-old) male Wistar rats after an iv injection of [^{3}H]Dt3 (0.03mg/100g body weight) for 2 hrs. The 2-hr. total biliary recovery of iv injected radioactivity (percent of the dose) was two times lower in old rats (7.40 ± 1.36% mean ± SD) compared with young rats (14.74 ± 4.10%). This difference was primarily due to the decrease in the excretion of Dt3 metabolites in the bile, while the excretion of the parent drug, Dt3 was 1.3 times higher in old rats. Among various Dt3 metabolites in the bile, digitoxigenin bis-digitoxoside (Dt2), digoxigen bis-digitoxoside (Dg2), and polar (conjugated) metabolites were major components, which all decreased with age. In accord with the decreased excretion of the radioactivity in the bile of old rats, the plasma disappearance of radioactivity was generally slower in old animals compared with young ones, yielding significantly higher plasma levels at different times of observation. Despite the increase in plasma radioactivity, the radioactivity concentration in the liver 2 hrs. after the injection was almost equal between the two age groups. It is suggested that at least in this rat strain and sex the biliary excretion of Dt3 metabolites was markedly age-dependent, presumably due to the decreased capacity of the liver to biotransform Dt3 with age. Furthermore, the lower liver plasma radioactivity ratio in old animals suggested the possibility that the distribution of Dt3 in the liver may also decrease with age.

INTRODUCTION

THE EFFECT of aging on the hepatic elimination of drugs has received less attention compared with renal elimination of drugs. Kato *et al.* (1964) reported that the hepatic metabolism of five different drugs decreased with age in parallel with their slowed plasma clearances in male rats. However, it was later recognized that this type of change cannot be observed consistently with other species, since Kato *et al.* (1970) reported no effect of age on metabolism of these drugs in mice. Many drugs which are metabolized by the liver are subsequently released as metabolities to the plasma and finally excreted by the kidney. There is another type of drug elimination which is mediated by means of biliary excretion. Digitoxin (Dt3) is known to be efficiently metabolized by the liver and its metabolites

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Abbreviations used: Dt3, digitoxin; Dt2, digitoxigenin-bis-digitoxoside; Dt1, digitoxigenin-mono-digitoxoside; Dt0, digitoxigenin; Dg3, digoxin; Dg2, digoxigenin-bis-digitoxoside; Dg1, digoxigenin-mono-digitoxoside; Dg0, digoxigenin; DMSO, dimethyl sulfoxide.

subsequently undergo an enterohepatic circulation in both man (Okita, 1957; Storstein, 1975) and rats (Klaassen, 1974). The effect of age on the elimination of drugs of this type has been little studied in the past. We previously reported that the biliary excretion of ouabain, another cardiac glycoside, is delayed with age in male Wistar rats (Kitani *et al.*, 1978), and suggested that there might be a transport mechanism for ouabain which is affected by aging; since ouabain is not biotransformed prior to its biliary excretion. In the present study we report the differences in the biliary excretion of Dt3 and its metabolites between 3-month and 25-month-old male Wistar rats.

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MATERIALS AND METHODS

Wistar derived male rats were purchased at the age of 11 weeks and were raised in the animal facility of the Institute with free access to food and water. Three animals were placed in a cage. Room temperature (23 ± 1°C), humidity (50 ± 5%) and lighting (6.00–18.00) were controlled. Filtered fresh air was continuously supplied by a special device (clean rack, Sanki, Tokyo) throughout the period of raising. In this condition, animals could be raised free from infection. The 50% survival time for this rat strain in these conditions was 26 to 27 months, which does not differ from data of our SPF aging colony. The main lesion found in later life in this rat was testicular tumor (interstitial cell tumor). Liver lesions such as periportal cell infiltrations, area or foci of varying degrees were also observed. Fatty degeneration, tumors of the liver and cystic lesions described as age-associated liver lesions in other rat strains (Burek, 1978) were of very low incidence. Three-month-old and 25-month-old rats were used for the study as representatives for young and old age groups.

Dt3 was obtained from Merk (Darmstadt, Germany). [^{3}H]Dt3 (generally labeled, 16mCi/mole), Protosol and Aquasol 2 were from New England Nuclear (Boston, Mass.). The radiochemical purity of [^{3}H]Dt3 examined by thin layer chromatography was over 98%; it was used without further purification. Dt2, Dt1, Dt0, Dg3, Dg2, Dg1 and Dg0 used as references for thin layer chromatography were obtained from Behringer (Manheim, Germany). Other chemicals for the extraction and separation of Dt3 metabolites in the bile were all of analytical grade. The experiments were performed between 9.00 a.m. and 3.00 p.m. The rats were allowed free access to food and water until use. All experiments were performed under a pentobarbital anesthesia (Nembutal, Abbott, North Chicago, Ill.). Rectal temperature was continuously monitored by a rectal probe and was maintained between 37° to 38°C by a heating lamp.

A femoral vein and artery were cannulated with PE-10 tubing for administration of Dt3 and blood sampling respectively. After an abdominal incision, the bile duct was cannulated with PE-10 tubing. After closing the abdomen, a 15-min. baseline bile sample was collected through this bile duct cannula. About 5μCi of [^{3}H]Dt3 with unlabeled carrier Dt3 dissolved in DMSO was injected through the femoral vein catheter. The dose of Dt3 was 0.03mg/100g body weight. After the injection, the bile was collected every 10 min. for 30 min., and then 30 min. and 1 hr. Arterial blood samples were obtained at 1, 3, 5, 10, 30, 60 and 120 min. after the injection of Dt3. During the 2-hr. experiment, a physiological saline was continuously infused through the femoral vein catheter to compensate for the loss of water by bile collection. After the experiments were over, rats were exanguinated and the liver, kidneys and heart were removed and weighed.

Bile flow rate was calculated from the weight of bile assuming the specific gravity of rat bile to be 1.0. Twenty μl of bile or plasma were mixed with 5 ml Aquasol 2 and the radioactivity was counted by a liquid scintillation counter (Aloka, Tokyo) using an automatic external standardization for quenching correction. Thirty to 100mg of tissue samples were weighed and mixed with 1ml of Protosol. After complete solubilization of tissue samples, 5 ml Aquasol 2 was added and mixed carefully. After keeping the sample in a refrigerator for a night, samples were subjected to the radioactivity determination. The standard solution was made from the original [^{3}H]Dt3 solution for injection with an appropriate dilution. Bile samples were frozen at −20°C for quantification of Dt3 metabolites which was performed later.

Twenty to 50 μl of bile sample was applied to a column packed with XAD-2 resin. Dt3 and its metabolites were eluted with 3 ml ethanol after washing the column with 6 ml distilled water. The ethanol fraction was evaporated and further subjected to thin layer chromatography, using DC Fertigplatten Kieselgel 60 (Merk, Darmstadt, Germany) after impregnation with 12.5% formamide (Storstein, 1976). After mixing the ethanol fraction with known amounts of unlabeled Dt3 and its metabolites, the sample was developed with ethyl methyl ketone: xylene (50:50) two times (Storstein, 1976). Each metabolite fraction was identified by corresponding Rf values of unlabeled metabolites which were developed in the same plate and localized by iodine vapor. Each radioactive fraction was separately scraped off into a glass vial and the radioactivity eluted by Aquazol 2. After a complete elution, the radioactivity was determined. The recovery of radioactivity by this entire procedure was over 90%. With assumptions that Dt3 and its metabolites were equally recovered from the bile, and that all metabolites had the same molar specific activities, the percent of each fraction to total radioactivity was calculated. Values of all metabolites were expressed as a weight of the parent drug Dt3 for practical convenience.

The statistical analysis was done with Student's *t* test. *P* values lower than 0.05 were considered to be significant.

RESULTS

In Fig. 1, the sequential changes in the bile flow rate, the biliary excretion of the total radioactivity (expressed as ng of the parent drug, Dt3), and its concentration in bile are shown for both young and old rats. In young rats, the bile flow rate was quite stable throughout the excretion study period. The biliary excretion rate of radioactivity increased with time in the first 30 min. and thereafter it was relatively constant. In old rats, a transient decline in bile flow rate was observed in the first 10-min. period. However, after this, the bile flow rate was also stable throughout the experimental period. Compared with young values, the bile flow rate expressed per 100g body weight was 20% lower in old rats. The concentration of radioactivity in the bile was also lower in old rats and the excretion rate of radioactivity was consistently lower in the older group. The cumulative biliary recoveries of total radioactivity as well as the excretion of Dt3 radioactivity are shown in Fig. 2 for both groups. The 2-hr. total biliary recoveries (percent of the injected dose) were 14.74 ± 4.10% (mean ± SD) in young rats and 7.40 ± 1.36% in old rats ($P < 0.001$), while the cumulative biliary excretion of the radioactivity of the parent drug, Dt3 was higher in old rats (see also Table 1).

Table 1 shows sequential biliary excretion of the parent drug, Dt3 and its metabolites in young and old rats. The Dt3 excretion rate was highest in the first 10 min. and then gradually decreased with time in both rat groups, while the excretion rates of its metabolites were all lowest in the first 10-min. and gradually increased with time. It is also clearly seen that the Dt3 excretion rate was lower in young rats compared with old ones in the most of the observation periods except for the first 10-min. period. Differences be-

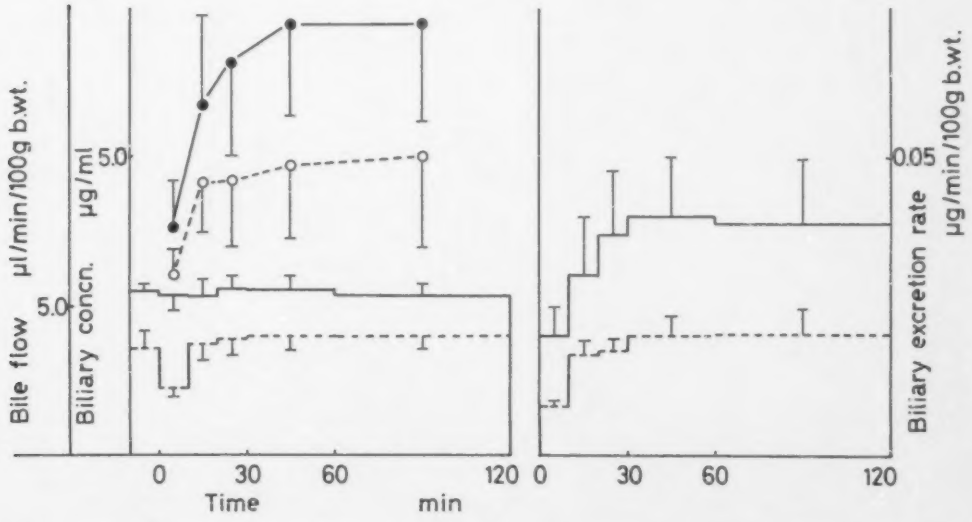

FIG. 1. Sequential changes in the bile flow rate (left panel), the biliary concentration (closed and open circles in the left panel) and excretion rate of radioactivity (right panel) in young (n = 6, solid line,) and old (n = 6, broken line) rats. All values were calculated as the weight of Dt3 and are expressed as mean ± 1S.D.

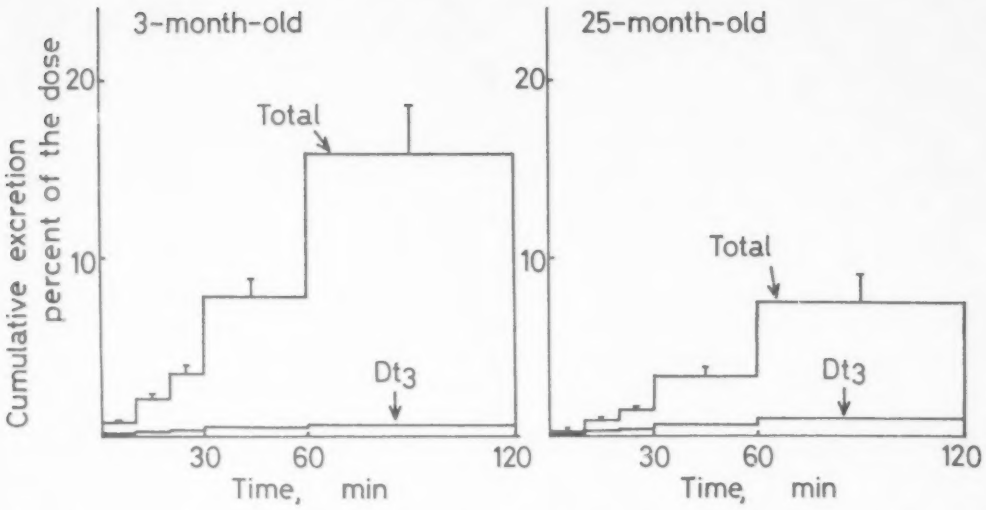

FIG. 2. Cumulative biliary excretion of total radioactivity and Dt3 radioactivity in young (solid line) and old rats (broken line). All values are expressed as mean ± 1S.D.

tween young and old values at periods from 10 to 120 min. were all significant ($p < 0.01$), although the difference in the average excretion rate for 2 hrs. did not attain a statistical significance. In contrast, the total excretion rate of metabolites was much lower in old rats compared with young rats. Thus, the age-dependent decrease in the biliary recovery of radioactivity was totally due to the decrease in the excretion rate of Dt3 metabolites. It is further seen that, in both age groups, the major metabolites of Dt3 in the bile were conjugated metabolites, Dt2 and Dg2, which can be better appreciated in Table 2. Compared with the excretion rates in the first 10-min. period, the excretion rates of most of the metabolites increased 2 to 3 times in the later periods of the experiment with the exception of Dg2 which increased 7 times in young animals and 10 times in old ones in the second hr. Consequently, the absolute excretion rates as well as the percentage to the total radioactivity in the bile of Dg2 and Dt2 were reversed as time elapsed with higher Dg2 values in later periods compared with higher Dt2 values in the first 10-min. period.

Table 2 shows the percentage of Dt3 activity relative to total bile radioactivity and that of each metabolite to total metabolite activity in both rat groups. It is clearly seen from Table 2 that the proportion of Dt3 to the total bile activity is 2 to 3 times higher in old animals compared with young ones.

Figure 3 further illustrates the relative excretion of individual metabolites and Dt3 in old animals in the 2-hr. period expressed as percentages of respective values of young animals. The excretion rate of metabolite in old animals ranged from 85% of Dg0 to 26% of Dg2 (and 35% of Dt2) of respective young values. In general, the decrease in the excretion of Dg related metabolites was slower compared with that of Dt related metabolites.

In Fig. 4, the disappearance of plasma radioactivity is compared between young and old animals. In old rats, the disappearance of plasma radioactivity was much delayed compared with young rats yielding significantly higher plasma radioactivities than young

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TABLE 1. SEQUENTIAL BILIARY EXCRETION OF DT3 AND ITS METABOLITES.

Time interval	Biliary excretion rate (ng/100 g/min)*					
	0–10	10–20	20–30	30–60	60–120	0–120
3-month-old (n = 6)						
Dt3	4.062 ± 0.866	2.487 ± 0.501	2.004 ± 0.462	1.524 ± 0.263	1.154 ± 0.265	2.246 ± 1.138
Dt2	3.013 ± 0.817	7.614 ± 1.897	8.877 ± 1.750	8.073 ± 1.163	6.286 ± 1.349	6.772 ± 2.490
Dt1	0.587 ± 0.106	0.892 ± 0.271	0.987 ± 0.251	0.994 ± 0.153	1.059 ± 0.297	0.903 ± 0.273
Dt0	0.730 ± 0.131	2.363 ± 0.679	3.452 ± 0.939	3.191 ± 0.775	2.445 ± 0.843	2.436 ± 1.181
Dg3	1.142 ± 0.344	2.011 ± 0.669	2.169 ± 0.375	2.241 ± 0.408	2.147 ± 0.566	1.942 ± 0.614
Dg2	1.710 ± 0.215	5.596 ± 1.164	8.997 ± 1.346	10.939 ± 1.893	11.788 ± 3.409	7.806 ± 4.188
Dg1	0.628 ± 0.165	1.038 ± 0.391	1.215 ± 0.387	1.285 ± 0.412	1.213 ± 0.393	1.076 ± 0.420
Dg0	1.041 ± 0.194	1.143 ± 0.239	1.137 ± 0.246	1.102 ± 0.215	1.011 ± 0.251	1.087 ± 0.255
Conj.	10.27 ± 1.810	15.37 ± 2.979	15.55 ± 3.410	14.90 ± 3.651	12.54 ± 3.415	13.725 ± 3.564
25-month-old (n = 6)						
Dt3	3.504 ± 1.051	3.815 ± 0.712	3.005 ± 0.328	2.699 ± 0.543	1.981 ± 0.494	2.960 ± 0.894
Dt2	0.733 ± 0.220	1.832 ± 0.637	2.212 ± 0.803	2.665 ± 1.065	2.669 ± 1.110	2.012 ± 1.039
Dt1	0.231 ± 0.050	0.380 ± 0.050	0.392 ± 0.055	0.453 ± 0.091	0.447 ± 0.122	0.381 ± 0.110
Dt0	0.186 ± 0.039	0.356 ± 0.044	0.471 ± 0.111	0.547 ± 0.167	0.623 ± 0.254	0.437 ± 0.207
Dg3	0.517 ± 0.145	1.209 ± 0.341	1.281 ± 0.269	1.461 ± 0.390	1.692 ± 0.819	1.232 ± 0.582
Dg2	0.406 ± 0.125	1.394 ± 0.461	1.985 ± 0.736	3.046 ± 1.225	4.560 ± 2.011	2.278 ± 1.787
Dg1	0.259 ± 0.144	0.477 ± 0.167	0.547 ± 0.172	0.628 ± 0.251	0.684 ± 0.187	0.519 ± 0.230
Dg0	0.569 ± 0.296	0.755 ± 0.494	0.928 ± 0.197	0.971 ± 0.218	0.993 ± 0.242	0.843 ± 0.330
Conj.	2.745 ± 0.904	6.655 ± 1.149	6.708 ± 0.832	6.629 ± 1.198	6.683 ± 1.226	5.884 ± 1.886

*All values are calculated as ng of Dt3 for convenience and expressed as mean ± 1S.D.

TABLE 2. RELATIVE PROPORTION OF DT3 AND ITS METABOLITES IN THE BILE.

Age Groups	3-month-old		25-month-old	
Time intervals (min)	0–10	0–120	0–10	0–120
Dt3	(17.62 ± 3.76)*	(7.00 ± 5.79)*	(38.70 ± 5.80)*	(20.56 ± 10.84)*
Dt2	15.69 ± 2.95**	18.59 ± 2.94	13.33 ± 3.17	14.32 ± 2.75
Dt1	3.07 ± 0.32	2.58 ± 0.42	4.24 ± 0.79	3.03 ± 0.76
Dt0	3.81 ± 0.37	6.44 ± 1.82	3.40 ± 0.59	3.19 ± 0.47
Dg3	5.97 ± 1.68	5.54 ± 1.31	9.24 ± 0.69	9.13 ± 1.83
Dg2	8.96 ± 0.48	20.35 ± 7.95	7.21 ± 0.90	23.94 ± 4.61
Dg1	3.30 ± 0.79	3.06 ± 0.90	4.58 ± 1.68	3.98 ± 1.41
Dg0	5.52 ± 1.15	3.33 ± 1.31	9.93 ± 2.68	7.03 ± 2.43
Conj.	53.68 ± 6.11	40.22 ± 9.76	48.08 ± 4.56	43.62 ± 9.34

*Values of Dt3 are expressed as percent of the total bile activity.
**Values of individual metabolites are expressed as the percent of total metabolite activity.
mean ± 1S.D.

values in most of the corresponding time periods. In Table 3, the percent of the injected radioactivity in the liver, liver plus bile, kidneys and heart, as well as the concentrations of activity examined at the time of sacrifice (2 hr. post injection), are summarized for both young and old animals. The percent distribution of radioactivity in the liver plus bile was significantly lower in old animals compared with young animals primarily due to the lower bile value in old animals. It is interesting to note that concentration of the liver

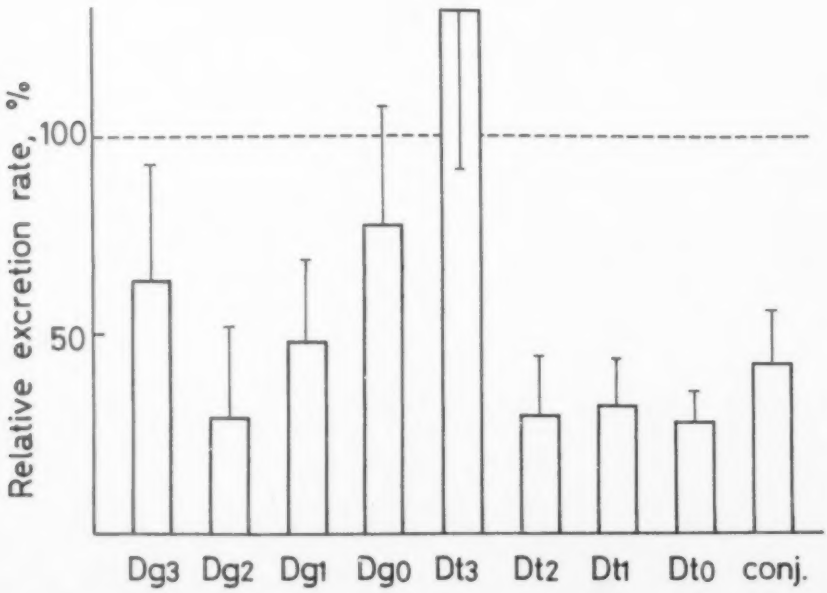

FIG. 3. Relative excretion of Dt3 and its metabolites in the bile in old rats in the 2-hr. period. Values are expressed as percent of the respective young values (mean ± 1S.D.).

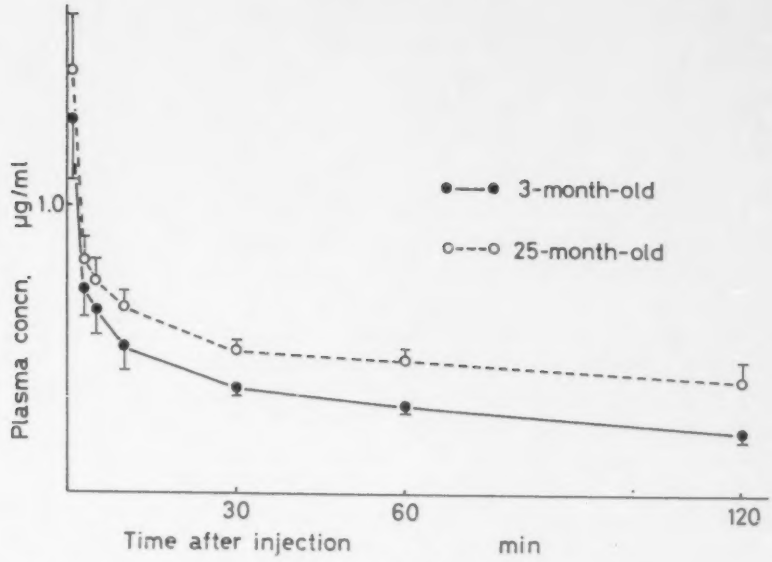

FIG. 4. Plasma radioactivity in young (solid line) and old rats (broken line) after [^{3}H]Dt3 injection. Values were calculated as the values for Dt3 and expressed as mean ± 1S.D.

TABLE 3. TISSUE DISTRIBUTION AND CONCENTRATION OF RADIOACTIVITY 2 HR AFTER INJECTION OF 3H DT3.

Age group	3-month-old	25-month-old
Body weight (g)	282.5 ± 8.3	501.7 ± 24.8*
Organ weight (g)		
Liver	8.10 ± 0.46	15.03 ± 1.45*
Heart	0.70 ± 0.07	1.07 ± 0.10*
Left kidney	0.87 ± 0.04	1.52 ± 0.10*
Right kidney	0.91 ± 0.04	1.50 ± 0.12*
Tissue radioactivity distribution (%) and concentration (μg/g)		
Liver	18.52 ± 1.96	17.74 ± 2.68
	(1.94 ± 0.35)**	(1.81 ± 0.44)
Bile (for 2 hr)	14.74 ± 4.10	7.40 ± 1.36*
Liver plus bile	33.26 ± 7.10	25.10 ± 4.10*
Heart	0.26 ± 0.07	0.38 ± 0.08*
	(0.31 ± 0.12)	(0.55 ± 0.12)*
Left kidney	0.32 ± 0.15	0.65 ± 0.13*
	(0.31 ± 0.15)	(0.65 ± 0.17)*
Right kidney	0.40 ± 0.12	0.59 ± 0.11*
	(0.37 ± 0.11)	(0.59 ± 0.14)*
Plasma	(0.22 ± 0.02)	(0.40 ± 0.07)*

All values are expressed as mean ± 1S.D. of animal experiments in each group.
*Significantly different from respective 3-month-old values ($p < 0.05$).
**Values in parentheses indicate radioactivity concentration expressed as μg of Dt3/g tissue.

radioactivity was not higher in old animals compared with young ones, while concentrations of plasma, heart and kidneys were all significantly higher in old animals at the time 2-hr. post injection. Consequently, the tissue plasma concentration ratio examined at 2 hr. post injection was almost identical between young and old animals for heart and kidneys, while liver plasma concentration ratio was considerably lower in old animals.

DISCUSSION

The biliary excretion study in the present experiment clearly demonstrated that the total recovery of radioactivity in the bile for 2 hr. decreased with age. This was primarily caused by the decrease in the biliary excretion rate of a variety of Dt3 metabolites. Dt3 itself was also shown to be excreted in the bile but, unlike its metabolites, its excretion was not decreased in old animals. Rather, the total excretion rate for 2-hrs. tended to be higher (1.3 times) in old animals compared with young ones. Although this difference did not attain a statistical significance due to the lower excretion in the first 10-min. period, the excretion rates of Dt3 during the periods from 10 to 120 min. were all significantly higher in old rats. This is most probably due to the higher availability of the parent drug Dt3 in the liver in old animals as a result of decreased biotransformation. The somewhat lower biliary excretion of Dt3 in the first 10-min. period in old rats may be related to the transient decrease in the bile flow observed in old rats or be due to the decreased initial distribution of Dt3 in the liver in old rats as discussed later.

Although we do not know the mechanism(s) involved in the biliary excretion of Dt3, it appears that it is not susceptible to the aging process. Thus, it is suggested that the biliary excretion process of Dt3 is different from that of ouabain for which a significant decrease with age was observed (Kitani *et al.*, 1978). If one assumes that the biliary excretion processes of Dt3 metabolites are as unaffected by aging as their parent drug Dt3, so that their biliary excretion is primarily dependent on the ability of the liver to biotransform Dt3, then the results of the present study suggest that in this rat strain and sex the hepatic metabolic capacity for Dt3 decreases considerably with age. This agrees with our observation on Dt3 biotransformation capacity for isolated hepatocyte preparation obtained from the same rat strain and sex, in which the velocity of Dt3 metabolism decreased 60% from 3 to 24 months of age (Ohta and Kitani, 1981).

As is also shown in our results, Dt3 is metabolized by the rat liver through several different metabolic pathways. Stepwise cleavages of the digitoxose sugar chain yield Dt2, Dt1, and Dt0, while 12β-hydroxylation can yield Dg3, Dg2, Dg1 and Dg0. Furthermore, considerable amounts of Dt3 metabolites are excreted in the bile in a conjugated form, which was reported to be mainly Dt1 conjugate with glucuronic acid (Castle and Lage, 1973). It was of interest to examine whether the appearance of some of these metabolites in the bile are differentially affected by aging. Results shown in Fig. 3, suggest that age affected the metabolism of Dt3 in general. However, when only unconjugated metabolites were compared, it appeared that Dg related metabolites were higher in proportion in old rat bile compared with young rat bile. Interestingly, in female BN/Bi rats, we found the opposite tendency yielding a higher proportion for Dt related metabolites in old animals (Kitani *et al.* 1982a). Thus, it seems that this differential effect on unconjugated metabolites by aging is mediated by sex hormone balance with age. In fact, the pattern of metabolite fraction of female Wistar rats resembles that of old males observed in the present study compared with young males (Kitani *et al.*, 1982b).

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As is shown in Fig. 4, the disappearance of plasma radioactivity was delayed in old animals yielding significantly higher specific activity in plasma throughout the observation period. Unlike ouabain which is taken up by rat liver by means of active transport (Eaton and Klaassen, 1978), the hepatic uptake of Dt3 was reported to be by means of diffusion.* Therefore, this higher level of Dt3 radioactivity is most probably due to the decreased biotransformation capacity of the liver and decreased biliary excretion.

It should be noted, however, that the hepatic concentration of radioactivity at 2 hr. post injection was almost equal between young and old animals. Since the biliary excretion of radioactivity was much lower in old animals, this suggests that initial distribution of radioactivity in the liver was lower in old animals. This was rather unexpected because the plasma radioactivity as well as concentrations in the heart and kidneys were all higher in old animals. Dt3 is very strongly bound to plasma protein, and probably to proteins inside the hepatocyte too. Furthermore, in the present study, only the total radioactivity in the liver and plasma but not that of each metabolite was determined. Thus, the free Dt3 concentrations in plasma and hepatocytes are difficult to predict from the information obtained in the present study. However, the data obtained in the present study suggest that the capacity to store Dt3 and or its metabolites by the liver decreased with age. Thus it is possible that this also contributed to the elevation of plasma radioactivity in old animals. In this regard, the study with female BN/Bi rats showed the radioactivity distribution as well as its concentrations in the liver examined at 2 hr. post injection was significantly lower in old rats compared with young ones (Kitani *et al.* 1982a) which essentially agrees with the present observation.

The projection of the results of the present study in terms of age effect on Dt3 metabolism should be done with considerable reservation, even for rats in general. Dt3 metabolism is known to differ greatly between male and female rats. The biliary excretion of Dt3 metabolites is 3 times higher in males (Kitani *et al.*, 1982b). Such a sex-dependent difference often leads to a significant sex difference in age-dependent changes. For example, the aminopyrine N-demethylation rate is 5 times higher in male rats compared with females at 6 months of age. But then while the demethylation rate decreased 5 times in males from 6 to 30 months, it decreased very little in female rats yielding very similar values at 30 months (Kitani *et al.*, 1981). In this regard, we recently performed a similar excretion study for Dt3 in female BN/Bi rats. Although the extent of the age-dependent decrease of biliary excretion was milder than observed in the present study, the biliary recovery of injected radioactivity decreased with age in female BN/Bi rats yielding 57% of the 3-month-old value at 30 months of age (Kitani *et al.*, 1982a). The higher plasma radioactivity, lower liver concentration and decreased metabolite excretion were all observed in old female rats compared to young females. Thus, the observations made in the present study may well be real age-dependent phenomena in rats, regardless of sex and strain.

However, the projection of results to human case again needs careful reservation. Although we have circumstantial evidences that in aged people the hepatic metabolism of Dt3 may be decreased with age (Sato *et al.*, 1976), we have had no direct proof.

Finally, the present experiment elucidated the metabolic and biliary excretory aspects of only the initial phase of Dt3 metabolism. In the clinical situation, with chronic treatment

*M. Schwenk, personal communication.

with Dt3, the steady state of Dt3 metabolism with a considerable enterohepatic circulation is usually maintained. It is also known that the final excretion of Dt3 metabolites is done via the kidneys. Thus, although the altered hepatic, metabolic and excretory capacity for Dt3 metabolites has been clearly demonstrated in the present study, their significance in the overall Dt3 pharmacokinetics in aged animals remains to be studied.

Acknowledgements—The authors wish to express their sincere gratitude to Mr. J. Ek who reviewed the manuscript. Thanks is also due to Mrs. K. Tagami and Mrs. T. Ohara who typed the manuscript.

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Experimental Gerontology, Vol. 17, pp. 417–423, 1982
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ENDOCRINE AGING IN CBA MICE: CHARACTERIZATION OF UTERINE CYTOSOLIC AND NUCLEAR SEX STEROID RECEPTORS

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(*Received* 21 *December* 1981)

Abstract—We examined the evidence of alterations with age in sex steroid hormone/receptor interaction in the uteri of CBA mice. The mean affinity for estrogens and progesterone that we observed in uterine cytosols and nuclei of old (16–18 months) mice were no different than those observed in young (4–8 months) animals. Whereas the total number of unoccupied estrogenic receptor sites in old uteri did not differ significantly from that observed in uteri of young mice, aging was accompanied by a 3-fold and significant decrease in mean nuclear binding sites when standardized as mg of DNA ($p < 0.05$). Contrary to estrogen receptors, aging uteri showed a 3-fold and significant decrease in the total number of uterine progesterone receptor concentration ($p < 0.05$) which was manifested by a decrease of progesterone binding sites in both the cytosol and the nucleus. These results suggest that aging CBA mice present a profile of altered hormone-receptor interraction which is only partly dependent on ovarian steroids.

INTRODUCTION

RESEARCH IN rodents has stressed the important role of extra-ovarian factors in general (Finch 1976 and 1979) and of altered hormone-receptor interraction specifically (Roth, 1979) in the etiology of reproductive aging. Yet, when studying the hormonal correlates of reduced fertility in CB strain of mouse, Holinka *et al.*, (1977) observed no significant impairment in uterotropic responses to estradiol with aging. Since this species of mice, contrary to most rodents, presents an endocrine profile of aging very similar to that observed in menopausal women (Collins *et al.*, 1980; Jones and Krohn, 1961; Nelson *et al.*, 1980; Papadaki *et al.*, 1979; Parkening, *et al.*, 1980), and considering the scarcity of data in that species on the evidence of alteration with age in sex steroid hormone/receptor interaction,

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Supported by a grant from the Medical Research Council of Canada

we undertook to further characterize that endocrine model of aging. This present report deals with our studies in the uterus of inbred CBA mice where cytosolic and nuclear sex steroid receptors were determined in relation to age.

MATERIALS AND METHODS

Hormones

Moxestrol, [11β-METHOXY-^{3}H] (R2858) [specific activity (SA): 70-90 Ci/mmol] and Promegestone [17α-METHYL-^{3}H] (R5020) [SA: 70-87 Ci/mmol] were obtained from New England Nuclear Corp., and purified by a Sephadex LH-20 column chromatography before utilization. The following unlabeled hormones were also purchased from New England Nuclear Corp.: R2858, R5020, 17β-estradiol (E_2), Diethylstilbestrol (DES), 5α-dihydrotestosterone (DHT), Testosterone (T), Progesterone (PRO), Cortisol (F) Methyltrienolone (R1881).

Chemicals and buffers

Organic solvents for the purification procedures and preparation of stock steroid solutions were freshly redistilled before use. The following buffers were used: A: 10 mM trishydroxymethylaminoethane-HCL, 1.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM dithiothreitol (DTT) at pH 7.4. B: buffer A to which 25 mM of $Na_2M_oO_4 \cdot 2H_2O$ (Sodium Molybdate) was freshly added before use. C: buffer A containing 10% glycerol (V:V).

Preparation of animals and tissues

Female CBA mice (2–4 weeks old) were purchased from Jackson Laboratory (Bar Harbour, Maine). They were housed in air-conditioned quarters and given food and water ad libitum. They were maintained in a colony (4–6 mice/cage) with careful monitoring of their chronological ages and their reproductive state was determined by serial vaginal smearing done at 6–8 months (pre-menopausal) or at 16–18 months (post-menopausal). Young female mice manifested at least 2 regular 4 day cycles before use and were studied in metestrous phase of their cycle. Most of the old ones showed persistent anestrus for 15 days prior to utilization, and careful inspection of their ovaries demonstrated atrophic changes and liquefaction compatible with their post-reproductive state. Seven groups (10–12 mice each) of young and old mice were sacrificed by decapitation between 800–1000 h and truncular blood was collected and pooled. The abdomen was then opened and the uteri were dissected, stripped of adhering fat and mesentery, and weighted. The blood was centrifuged and kept frozen until assayed, whereas the uteri were rapidly covered with saline and immersed in crushed ice for immediate processing. Post-menopausal mice presenting pathological signs of aging as determined by macroscopic inspection of the liver, the kidneys, the spleen and the bowels were excluded from the study. All tissue manipulations were subsequently performed at 4°C either in a cold room or on crushed ice.

Cytosolic and nuclear extracts

Uteri were minced and washed (×3) in buffer A. The suspension (1 gr/4 ml) was then homogenized with a Polytron (PT-10). Buffer B or C were then added to the homogenate for stabilization of estrogen and progesterone cytosolic receptors respectively. The homogenate was then centrifuged at 600 g × 10 min. at 4°C. Cytosols were obtained as the supernatant fraction after further centrifugation at 4°C at 105,000 g × 60 min., whereas nuclei were obtained by suspending the 600 g. pellet in buffer A modified to contain 2.5 M sucrose and centrifugation at 600,000 g × 60 min. at 4°C. The purified nuclear precipitate was then extracted with 0.4 M KCl and further centrifuged at 105,000 g × 60 min. Preliminary experiments with CB mice pre-treated with [^{3}H] R2858 or [^{3}H] R5020 showed an extraction efficiency better than 75% in both age groups.

Cytosolic and nuclear receptors

Unoccupied uterine cytoslic receptors were measured according to modified methods of Jordan *et al.* (1978) for estrogens and of VU HAI & MILGROM (1978) for progesterone, using R2858 and R5020 as respective ligands. Unoccupied nuclear receptors were measured by adapting a method currently in use in our laboratory for determination of nuclear androgen receptors in human prostate (Lehoux *et al.*, 1980). Separation of the free hormone from the hormone receptor complex was done by DEXTRAN-COATED CHARCOAL (DCC) and the graphic representation of SCATCHARD (1949) was used for the saturation studies as well as the determination of affinity constants and the number of binding sites. Protein determination was done by the method of Lowry

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(1951) with BSA as a standard; DNA determination was measured in the residual KCl-extracted nuclear pellets (procedural losses ≤ 15%) according to the method of Burton (1956).

Hormone determination

Serum E_2 and PRO levels were determined by specific radioimmunoassays (RIA) as previously published by our laboratory (BELISLE, 1979). For mice assays, the sensitivity was 10 pg/ml for E_2 and 0.25 ng/ml for PRO, and the intra and inter assay variability did not exceed 15%. When possible all pooled specimens were measured in the same RIA.

Statistics

Analysis of data for levels of significance was done by the student's *t* test.

RESULTS

Validation of steroid receptor assays

Incubation conditions. Extracts (50 λ) from the cytosols and the nuclei from both age groups were incubated in duplicate aliquots with a saturating dose (20×10^{-9}M) of either tritiated R2858 or R5020 for 2, 4, 18, 24 hours and at 4° and 22°C. Total binding sites were maximally saturated at 4°C by 18 hours in the cytosols and by 4 hours in the nuclei and longer incubation time produced no significant change in binding. All incubations were subsequently done for 18 hours (overnight) at 4°C.

Saturability and specificity. Extracts (50 λ) from the cytosols and the nuclei of uteri obtained from both age groups were incubated in the presence of 1×10^{-9}M tritiated R2858 or R5020 and increasing concentrations ($0–100 \times 10^{-9}$M) of the corresponding radioinert ligand to obtain saturation curves. Non-specific binding was corrected by parallel incubation with 100-fold excess of either DES or F. Saturation of the specific R2858 binding sites was reached at a hormone concentration of 15×10^{-9}M for the cytosols and of 12×10^{-9}M for the nuclei. Similarly, for R5020 binding sites, leveling off of the curve was observed at 20×10^{-9}M in the cytosols and at 10×10^{-9}M in the nuclei. In contrast to uterine tissues, binding of R2858 or of R5020 to plasma increased linearly and no saturation was observed. Uterine cytosolic and nuclear extracts were also incubated with a mixture of either tritiated R2858 or R5020 and various unlabeled hormones at increasing concentrations ($0–1000 \times 10^{-9}$M) for competition studies. The binding of [^{3}H] R2858 (100%) was easily displaced by DES (110%), E_2 (90%), and was not affected significantly by R5020, DHT, F, PRO, R1881 and T. Similarly, the binding of [^{3}H] R5020 (100%) was easily displaced by PRO (80%) but not by the other ligands.

Sucrose density gradient. Cytosolic and nuclear fractions from both age groups were incubated overnight at 4°C in the presence of 10^{-9}M [^{3}H] R2858 or [^{3}H] R5020 a) without any addition of radioinert R2858 or R5020 and b) in the presence of 10^{-6}M unlabeled R2858 or R5020. The complexes were then separated by DCC and layered on a 5%–30% continuous sucrose density gradient prepared in buffer A. After further centrifugation at 230,000 g × 18 hours with a SW-50 rotor (BECKMAN), the bottom of the tubes were punctured and fractions counted. Sedimentation profiles of cytosolic binding sites were in the 8 S area for both R2858 and R5020, whereas those of nuclear binding sites were in the

TABLE I. MEAN ± S.E. AFFINITY CONSTANTS (K_a) AND CONCENTRATIONS (n) OF ESTROGEN AND PROGESTERONE UTERINE RECEPTORS IN PRE AND POST MENOPAUSAL CBA MICE.

	PRE-MENOPAUSAL (N = 84)		POST-MENOPAUSAL (N = 78)	
	K_a ($\times 10^9 M^{-1}$)	n (fmol)*	K_a ($\times 10^9 M^{-1}$)	n (fmol)*
ESTROGEN [RANGE]				
CYTOSOL	0.72 ± 0.17 [0.38 – 1.70]	217.6 ± 22.7 [137.9 – 301.4]	0.75 ± 0.13 [0.54 – 0.97]	292.6 ± 90.2 [181.5 – 385.4]
NUCLEUS	0.93 ± 0.19 [0.84 – 1.71]	186.6 ± 57.6 [73.9 – 373.7]	0.86 ± 0.16 [0.79 – 1.24]	56.7 ± 11.6 [30.1 – 68.3]
PLASMA E_2 (pg/ml)	32.5 ± 3.8		<10	
PROGESTERONE [RANGE]				
CYTOSOL	0.47 ± 0.11 [0.32 – 0.77]	501.8 ± 95.1 [295.7 – 706.8]	0.29 ± 0.08 [0.21 – 0.44]	167.6 ± 133.2 [36.4 – 437.9]
NUCLEUS	0.71 ± 0.27 [0.17 – 1.61]	398.6 ± 157.4 [163.8 – 577.9]	0.42 ± 0.12 [0.26 – 0.59]	80.8 ± 16.3 [56.7 – 105.8]
PLASMA PRO (ng/ml)	11.6 ± 1.1		1.9 ± 0.3	

*n standardized as mg of protein for cytosol and as mg of DNA for nucleus.

5 S area. All complexes were completely saturated by the addition of 10^{-6}M of the corresponding radioinert ligand.

Characterization with aging

At the time of the experiment, the average weight of young animals (18 gr) was no different than that observed in old ones (22 gr). Similarly, the mean ± S.E. whole uterine weight did not change significantly with aging (142.0 ± 16.0 mg vs 116 ± 13.0 mg). Scatchard analysis of the data from 7 groups of young (84 mice) and old (78 mice) animals are represented in Table I, and FIGS. 1 and 2 illustrate representative examples of those studies.

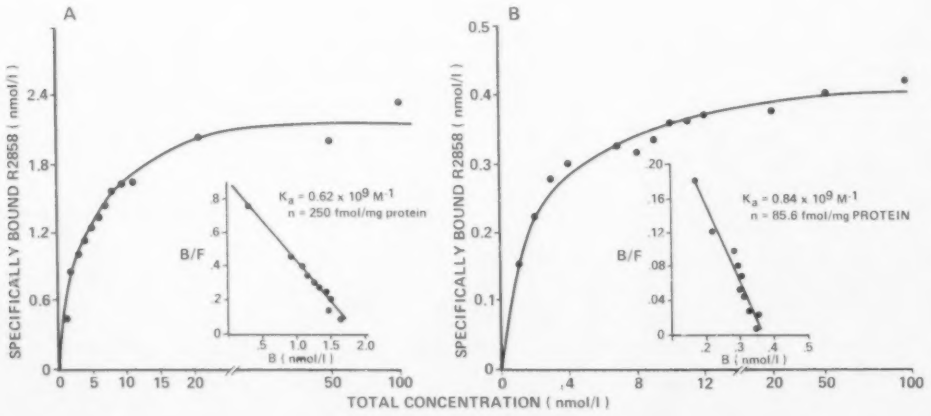
A
B
SPECIFICALLY BOUND R2858 (nmol/l)
B/F
B (nmol/l)
K_a = 0.62 x 10^9 M^{-1}
n = 250 fmol/mg protein
K_a = 0.84 x 10^9 M^{-1}
n = 85.6 fmol/mg PROTEIN
TOTAL CONCENTRATION (nmol/l)

FIG. 1 shows a representative example of a saturation study with its Scatchard representation of estrogen binding to cytosolic (A) and nuclear (B) extracts in the uterus of CBA mice.

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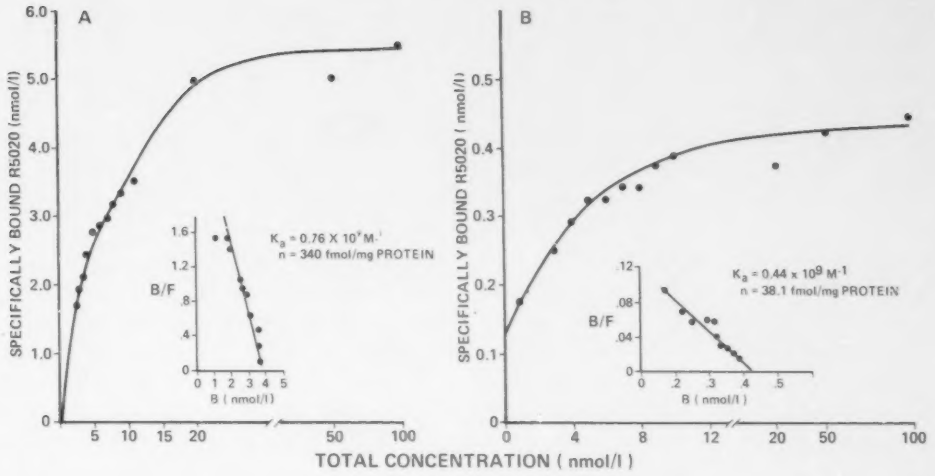

FIG. 2 shows a representative example of a saturation study with its Scatchard representation of progesterone binding to cytosolic (A) and nuclear (B) extracts in the uterus of CBA mice.

Extracts from uteri of young reproductive CBA mice contained a single class of binding sites with a mean Ka for estrogens of 0.72 ± 0.17 (S.E.) × 10^9M^{-1} in the cytosol and of 0.93 ± 0.19 × 10^9M^{-1} in the nucleus. The mean Ka of 0.75 ± 0.13 × 10^9M^{-1} and of 0.86 ± 0.16 × 10^9M^{-1} for estrogens that we observed respectively in uterine cytosols and nuclei of post-reproductive mice were no different than those observed during the reproductive state. On the other hand, after the onset of menopause, while the number of specific estrogenic binding sites did not change significantly in the cytosols [217.6 ± 22.7 *vs* 292.6 ± 90.2 fmol/mg protein, $p > 0.05$], a 3-fold and significant decrease in the mean nuclear binding sites for estrogens was observed [186.6 ± 57.6 *vs* 56.7 ± 11.6 fmol/mg DNA, $p < 0.05$].

For progesterone binding sites, scatchard analysis in young CBA mice showed a mean ± S.E. Ka of 0.47 ± 0.11 × 10^9M^{-1} in uterine cytosols and of 0.71 ± 0.27 × 10^9M^{-1} in nuclear extracts. Although aging did not significantly change the affinity for progesterone to its binding sites, old mice showed a 3-fold decrease in uterine cytosolic receptors (501.8 ± 95.1 vs 167.6 ± 1332. fmol/mg protein $p < 0.05$) and a 5-fold and significant decrease in nuclear progesterone binding sites (398.6 ± 157.4 vs 80.8 ± 16.3 fmol/mg DNA $p < 0.05$).

Whereas the total number of unoccupied estrogenic receptor sites of 16.8 ± 2.4 fmol in post-menopausal uteri, as determined by the sum of the cytosolic and nuclear binding sites per mg of uterine weight, did not differ significantly from that of 18.1 ± 3.2 fmol observed in uteri of pre-menopausal mice, aging was accompanied by a 3-fold and significant decrease in the total number of progesterone receptor concentration (43.4 ± 2.8 US 15.1 ± 4.6 fmol/mg uterine weight $p < 0.01$).

DISCUSSION

Our investigation confirmed the presence in the uteri of CBA mice of proteins which possessed properties expected of a hormone receptor. This is based on the finding in both

the cytosol and the nucleus of macromolecules which combined the characteristics of specific, high affinity—low capacity binding sites, with appropriate sedimentation profiles. Our mean affinity constants observed for estrogen receptors agree with those reported by Aihara *et al.*, (1980) and by Papadaki *et al.*, (1979) in uteri of young and old CB strain of mouse respectively. Our study is the first to characterize cytosolic and nuclear progesteronic receptors in the uterus of CB strain of mouse, and our observations agree with those reported for uteri of other strains of mice (Feil *et al.*, 1972; KORACH, 1979).

The main finding of our study is that aging is not accompanied by any changes in the affinity, saturability, specificity or the sedimentation profile of estrogens and progesterone binding sites in uteri of CBA mice. Furthermore, no age difference was observed in the total number of estrogen binding sites. This is contrary to what was observed in other rodents but agrees with preliminary work of Gosden (1976) in a similar strain of mouse which showed that the levels of radioactive estradiol uptake in whole uterine extracts did not change with age. Our results also suggest that, with advancing age, a shift in the intracellular distribution of estrogenic receptors occurs, more binding-proteins being observed in the uterine cytosols than in the nuclei. This conclusion is contrary to the work of Gesell and Roth (1981) in aging rats, but agrees with that of Flickinger *et al.*, (1977) in post-menopausal women. Although few studies exist on the concentration of uterine steroid receptors in aging CBA mice, the number of estrogenic binding sites that we observed in cytosolic and nuclear extracts of old mice agreed well with those reported by others in aging (Papadaki *et al.*, 1979) or ovariectomized (Aihara *et al.*, 1980) CBA mice, thus adding validity to our observations. Several possibilities could explain this apparent intracellular redistribution of sex steroid receptors with spontaneous aging. Thus, as circulating estradiol decreases with aging, much less steroid would interact with its specific receptor to translocate into the nucleus, and the sex steroid receptors would remain primarily localized in the cytosolic fraction of the cell. We would favor this explanation since the concentration of sex steroid receptors and their intracellular compartmentalization are partly under the control of endogenous hormonal stimuli (Vu Hai *et al.*, 1977; Feil *et al.*, 1972; Martel & Psychoyos, 1978; Cidlowski & Muldoon, 1978; Walters & Clark, 1979; Papadaki *et al.*, 1978; Aihara *et al.*, 1980). This hypothesis is further enhanced by our observations with progesterone receptors, where a threefold reduction in total progesterone binding sites paralleled the threefold reduction in nuclear estrogen receptors observed in our group of post-reproductive mice.

Since the significant decrease in circulating plasma E_2 that we observed in old CB mice was not paralleled by a concomitant decrease in the total number of uterine estrogen binding sites, an alternative explanation for the shift in cytosolic/nuclear distribution of those receptors would be that aging impairs the ability of uterine sex steroid receptors to translocate from cytosol to nucleus. Such a biological inactivation of estradiol receptor with age has already been observed in the brain and pituitary of old female rats (Jiang and Peng, 1981) as well as in the uteri of mice (Korach, 1979). Further studies are in progress in our laboratory to resolve this hypothesis which might lead to a better understanding of the induction of menopause in mammals.

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Experimental Gerontology, Vol. 17, pp. 425–428, 1982
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DYING OLD MICE: OCCURRENCE OF NON-VIABLE LYMPHOCYTES AND AUTOAGRESSIVE CELLS

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(*Received* 4 *June* 1981)

Abstract—Sixteen to twenty-two months old untreated CBA and C57/Bl mice judged to die within 24 hours had a higher percentage of dead leukocytes in lymph nodes, spleen and thymus than had like-aged old mice and young (2–8 months) healthy mice. Furthermore, dying CBA and DBA mice showed enhanced *in vitro* cytotoxicity of spleen lymphocytes towards syngeneic fibroblasts and YAC target cells.

INTRODUCTION

BOTH THE events precipitating the process of dying and the initial bodily changes can be of a very different nature. However, irrespective of initial change there may well emerge a process common to most dying and consequently of interest for those cases where medical intervention is desirable.

MATERIALS AND METHODS

Untreated inbred, specific pathogen free CBA, C5 and DBA female *mice* (Staats, 1972) of 2 and 16 months-of-age were used. All animals were inspected 6 days a week for 6 months, and when one of the old mice were judged to die within 24 hours that animal plus another old but healthy looking mouse and a young mouse were sacrificed by cervical dislocation and cells harvested immediately for dye exclusion tests. Cytotoxicity testing was carried out whenever there were 3 dying mice on the same day. For all mice lung, liver, spleen, kidney, thymus, thyroid and peripheral and mesentheric lymph nodes were taken for microscopy and their gross pathology recorded.

For the supravital dye exclusion test lymphoid organs were removed and immediately placed in RPMI-1640 with 10% heat inactivated fetal calf serum on ice. After cutting the organs with a razor blade, and shaking the suspension gently, 2 minutes were allowed for clumps to settle before 1 ml monocellular suspension was mixed with a similar volume of 2% nigrosin in buffered saline on ice and live and dead lymphocytes counted (Claesson, 1975). The nigrosin used in our hands gave lower values for dead lymphocytes than test with trypan blue. Smears, later to be Giemsa stained were also made. Granulocytes which comprise about 25% of viable lymph node cells, 18% of spleen cells and 9% of thymus cells were excluded from total counts. Dying granulocytes were likewise excluded when dying lymphocytes were counted. In all tests we also diluted 10^6 leukocytes in more medium to a final volume of 10 ml, and kept them at 37°C, 5% CO_2 for 24 hours. Thereafter, the total lymphocyte cell count and percentage nigrosin stainable lymphocytes were determined. Furthermore thymocytes and spleen cells were kept at 37°C in undiluted serum from young mice and in serum of old dying mice of the same strain for 30 min. prior to nigrosin test for viability.

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Sponsored by The Danish Cancer Society.

Test for autoreactivity. (Olsson and Ebbesen 1979) Syngeneic embryo fibroblasts and the A strain lymphoma YAC-1 cells (Haller et al., 1977) were used as target cells. After one wash in RPMI the pellet of 5×10^6 cells had 200 μCi ^{51}Cr and .8 ml RPMI added. After one hour of wagging at 37°C, 5% CO_2 the cells were washed three times in phosphate buffed saline, and finally made up to 10^5/ml in RPMI. Each microliter plate well received .1 ml of target cell suspension.

Spleen cells obtained from tumor free spleens were washed once in RPMI and resuspended in 4 ml RPMI, and subsequently layered upon 3 ml Ficoll-isopaque (12 parts 14% Ficoll to 5 parts 33% isopaque), and centrifuged 15 min 2000 g at 20°C. After the lymphocyte layer had been harvested it was washed once in 5 ml RPMI and then resuspended in RPMI. The live cells were counted and made into a suspension containing 10^6 live cells per ml. This procedure removes red cells, monocytes and granulocytes leaving 90% of the original lymphocyte population with a 3% ad mixture of granuiocytes. To each microtiter .1 ml of the lymphocyte-suspension was added well on top of the target cells. Lymphocytes from each mouse were added to 24 wells with labelled target cells. In addition, target cells were incubated with no attaching cells to give spontaneous release and labelled target cells were treated with water to give total cronium release. The microtiter plate was kept on a wagger at 37°C, 5% CO_2 for 4 hours. Thereafter, the supernatant was harvested and counted in a gamma counter.

Three dying old mice, 3 healthy old ones and 3 young mice of one strain were always tested together. Spontaneous isotope release from target cells was slightly lower than the smallest release with lymphocytes from any of the three subgroups tested.

The result of each days testing was expressed as $y - x/\text{Total} - x \cdot 100\%$. x is the smallest mean release obtained with lymphocytes from one of the three subgroups on that particular day. y is mean value for another group. Total is the release obtained by incubating the labelled cells with water.

RESULTS AND DISCUSSION

Of 76 mice deemed dying but deliberately not sacrificed 56 were found dead the following day, and allowing two days after diagnosis 70 out of the 76 mice were gone. Of the 3000, 16 months-old animals entering the study 24% were dead 6 months later. During the study period there were 26 cases where a mouse judged not to be dying was nevertheless found dead the next morning. Dying animals weighted about 10% less than old, healthy looking mice.

The mean value for the percentage of dead lymphocytes in cell suspensions studied immediately after sacrifice was higher in dying than in other mice. Standard deviations were, however, high so the *t*-test indicated no difference of mean at the 1% level. Spermans rank test indicated a difference between values for lymph nodes and spleens in CBA and large thymus cells in C57B1 thymus (Table 1). Most dying mice had histologic evidence of either reticulosarcoma or mammary tumor. Presence or absence of these malignancies did not seem to influence the percent of dead lymphocytes. Incubation for 30 min in undiluted mouse serum caused no detectable cell killing irrespective of whether serum from you or old dying animals was used. Twenty-four hours incubation in medium killed 50–80% of the cells whether harvested from young or dying animals.

Dying is here used to encompass processes that lead to the death of the mice within a few days. Different initiating events might cause chain reactions that converge on some processes common to many dying animals. Acting on that assumption we looked for death of lymphoid cells prior to clinical death and for evidence of self-destructive behavior of lymphoid cells.

Although our test animals may start dying for many reasons and must be at different time distances from spontaneous death at the time of sacrifice, we found a general tendency for cell suspensions made from dying animals to hold more dead lymphocytes than healthy mice of the same age. We found no difference in fragility between lymphocytes from the various groups as measured by 24 hour *in vitro* survival and no evidence that serum from old dying mice is *in vitro* toxic to lymphocytes from old or young mice. As no

TABLE 1. MEAN PERCENTAGE DEAD LEUKOCYTES IN LYMPHOID ORGANS IMMEDIATELY AFTER SACRIFICE.*

Strain	Subgroup	Number of mice	Lymphnodes	Spleen	Thymus	
					Large cells (>10μ)	Small cells
CBA	young	42	10	3,8	6	0
	old healthy	36	2,6	1,2	7	2
	dying old (all)	47	12 P2 < 0.01	4 P2 < 0.01	12	2,5
	dying no tumor	10	12	3,3	20	4
C57Bl	young	15	2,6	1,7	3	1
	old healthy	17	7	3,7	5	5
	dying old (all)	33	10	5,3	12 P2 < 0.01	2
	dying no tumor	11	6,3	2,8	14	1
DBA	old healthy	40		2,4		
	dying old	36		3,5		

*Test for difference between dying and healthy old animals at the 1% level was done with *t*-test for difference of mean (P1) and with Spermans rank test (P2).

kinetic studies were made we do not know if the enhanced percentage of dead lymphocytes in dying animals is due to increased rate of leukocyte dying or decreased rate of removal of dying cells. Also the distribution of decay on the many functional subpopulations of lymphocytes is unknown. An increased percentage of dead lymphocytes was found irrespective of the animals showing histologic evidence of malignancy or not, suggesting that lymphocyte decay is common to death brought about by several causes.

Our observation of enhanced autoreactivity towards syngeneic fibroblasts and of enhanced cytotoxicity towards YAC-1 cells that are much in use as target for natural killed cells (Herbermann et al., 1975) suggests that self-destruction brought about by cytotoxic lymphocytes (Ono et al., 1977) can be part of the process of dying (Table 2).

Autoreactivity could be precipitated by infection with subsequent release of interferon which activates NK-activity (Huddlestore, Merigan and Oldstone, 1979), or by a preferential decay of suppressor T-lymphocytes (Cohen and Wekerle, 1973), but this is purely speculative. It is, however, known that healthy aging mice (Walford, 1974) and elder humans (Makinodan and Yunis, 1977) often show evidence of low level autoreactive processes. Human death associated with cancer and cardiovascular disease is furthermore reported to be more frequent among those with serum antinuclear antibodies than among those without (Roberts-Thomsen et al., 1974).

TABLE 2. CELLULAR CYTOTOXICITY OF SPLEEN LYMPHOCYTES TOWARDS SYNGENEIC FIBROBLAST AND YAC CELLS.*

			Target cells			
			Fibroblasts		YAC cells	
Strain	No. of groups (3 young, 3 old healthy, 3 dying old)	Sub-group	No. of sub-group with highest count in each group	% increase (mean)	No. of sub-groups with highest count in each group	% increase (mean)
		young	1	Baseline	1	Baseline
CBA	18	old healthy	5	9	2	19
		dying old	12	13	15	32
			$p < 0.01$		$p < 0.01$	
		young	1	Baseline	1	Baseline
DBA	18	old healthy	3	5	3	5
		dying old	14	10	14	41
			$p < 0.01$		$p < 0.01$	

*Test for difference between dying and healthy old animals was done with chi-square test.

Acknowledgements—This work was supported by The Carlsberg Foundation and NIH grant 1R01 CA 26109-02.

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Experimental Gerontology, Vol. 17, pp. 429–435, 1982
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EFFECT OF INHIBITORS AND STIMULATORS ON ISOLATED LIVER CELL MITOCHONDRIAL PROTEIN SYNTHESIS FROM YOUNG AND OLD RATS

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(*Received* 18 *January* 1982)

Abstract—Mitochondria isolated from the livers of old rats (26–30 months) were found to incorporate 41% less leucine into mitochondrial proteins as compared to those from young rats (2–3 months). The initial rates of incorporation of label were 145 cpm/mg/min for the "old" animals, and 320 cpm/mg/min for the young animal. No difference in either amino acid pool size or leakage of label through the mitochondrial membrane was detected in the two age groups. Young rats were treated *in vivo* with cycloheximide (10 mg/kg) followed by isolation and incubation of their mitochondria *in vitro* two hours later. There was a two-fold increase in incorporation of leucine into mitochondrial proteins. In contrast, mitochondria isolated from old rats showed a markedly blunted response to cycloheximide pre-treatment. When mitochondria isolated from young and old rats were exposed to inhibitors of mitochondrial protein synthesis, α-α-dipyridyl (2×10^{-4}M) and ethanol (0.15M), the old mitochondria showed greater susceptibility to inhibition. These results suggest that the control of the biosynthesis of mitochondrial proteins is altered in the old animals.

INTRODUCTION

MITOCHONDRIA CONTAIN their own unique protein synthesizing system which resembles that in prokaryotic cells (Ibrahim and Beattie, 1976; Lin *et al.*, 1974; Marcus *et al.*, 1980; Mason and Schatz, 1973; Tzagaloff *et al.*, 1973; Weiss, 1972). This system is under the control of mitochondrial DNA and produces the hydrophobic structural proteins of the inner mitochondrial membrane (Hawley and Greenawalt, 1970; Ibrahim and Beattie, 1976; Ibrahim *et al.*, 1973; Marcus *et al.*, 1980; Schatz and Mason, 1974). Under normal growth conditions there must be coordinating signals between the cytoplasm and mitochondria in order to maintain balanced synthesis (Marcus *et al.*, 1980). We have previously postulated that this might occur via cytoplasmic proteins such as the hemin-controlled repressor (Freedman *et al.*, 1974; Freedman and Rosman, 1976) or heme or iron concentrations (Marcus *et al.*, 1980).

It has been shown that there is a decreased synthesis of cytoplasmic proteins in liver cells from aged rats as compared to cells from young ones (Moldave *et al.*, 1979). If an

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age-related decrease in mitochondrial protein synthesis is a reflection of a decline in cytoplasmic protein synthesis, pre-treatment of young rats with cycloheximide, an agent which inhibits cytoplasmic protein synthesis but does not affect isolated mitochondrial protein synthesis (Marcus *et al.*, 1980), should mimic the aging lesion and decrease mitochondrial protein synthesis. Our results, however, indicate that cycloheximide pre-treatment of young rats *in vivo* results in the opposite effect in that there is an increase in isolated mitochondrial protein synthesis. In the old rats this phenomenon was markedly blunted showing that the decline in mitochondrial protein synthesis with age is not merely a simple reflection of the decline in cytoplasmic protein synthesis. Furthermore, upon treatment of isolated mitochondria from old and young rats with inhibitors of mitochondrial protein synthesis, α,α-dipyridyl (Marcus *et al.*, 1980) and ethanol, a greater inhibition was seen with both of these agents in the old animals' mitochondria demonstrating a greater susceptibility to inhibition.

METHODS AND PROCEDURE

Mitochondria were isolated from the livers of young (6–12 weeks) and aged (112–130 weeks) male Sprague-Dawley rats by methods previously described (Marcus *et al.*, 1980). Those rats pre-treated with cycloheximide were injected i.p. with 10 mg/kg body weight in saline two hours prior to sacrifice. Controls received an equal volume of saline.

Incubations of the isolated mitochondria were as previously described (Marcus *et al.*, 1980) for 30 minutes at 37°C in shaking water bath. In experiments measuring incorporation of radioactive amino acid into mitochondrial protein the precursor used was L-(U-^{14}C) leucine, 15μCi/ml. Each 2 ml incubation flask contained 15μM radioactive leucine and 2–3 mg mitochondrial protein. Additions were made as shown in the tables. Incubations were terminated by addition of 10mM non-radioactive L-leucine followed by precipitation of protein with 15% trichloroacetic acid. The labeled proteins were washed and counted as previously described (Marcus *et al.*, 1980). Protein concentration was determined by the method of Lowry *et al.* (1951).

Cycloheximide was obtained from the Sigma Chemical Company; α,α-dipyridyl from Fisher Scientific Company; L-(U-^{14}C) leucine, 350 Ci/mol, from Amersham. All other reagents were of the highest grade commercially available.

RESULTS

In order to show that we were indeed measuring isolated mitochondrial protein synthesis the following safeguards were taken: (1) sterile incubations; (2) periodic sampling for bacterial culture; (3) tubes minus mitochondria were incubated and processed to show that incorporation does not occur in the absence of mitochondria (Marcus *et al.*, 1980). In addition, blood samples were taken from the animal at the time of sacrifice to exclude iron deficiency as a cause of diminished mitochondrial protein synthesis (Marcus *et al.*, 1980).

The effect of age on isolated rat liver mitochondrial protein synthesis is shown in Table 1. There was a mean 41.2% decrease in mitochondrial protein synhesis in those organelles isolated from old animals as compared to those from young rats. The size of the leucine pool in mitochondria from young and old rats was also measured. Mitochondria were incubated with L-(^{14}C) leucine for 30 minutes as described in Experimental Procedures, washed with isolation media (5 volumes) until the washings were free of counts, lysed by the addition of 2 ml 5% TCA, and the amount of radiolabel in the supernatants and quantity of mitochondrial protein determined. There were 876 cpm/mg and 784 cpm/mg released by the old and young mitochondria respectively. Under the conditions of this assay the old rat mitochondria incorporated 5341 cpm/mg as compared to 8772 cpm/mg in the young. Mitochondria from young and old rats were incubated with L-(^{14}C)

TABLE 1. THE EFFECT OF AGE ON INCORPORATION OF L-(U-^{14}C) LEUCINE INTO PROTEIN OF ISOLATED RAT LIVER MITOCHONDRIA INCUBATED *IN VITRO* (n = 8)[a].

Young (2–3 months)	Old (26–30 months)
cpm/mg mitochondrial protein ± SEM	cpm/mg mitochondrial protein ± SEM
6687 ± 233	3932 ± 327[b]

[a]Incubations were as described in Materials and Methods for 30 minutes at 37°C in triplicate with less than 10% variation between samples.

[b]Old statistically differs from young by Student *t*-test analysis ($p < 0.001$).

leucine for 30 minutes as described in Experimental Procedures, washed with isolation medium and reincubated with non-radioactive leucine for an additional 30 minutes. No significant leakage of the label into the medium from either the old or the young mitochondria was found.

The time course of incorporation of label into mitochondrial protein for the young and old animals is shown in Fig. 1. The young animal used in this experiment was 8 weeks old while the old animal was 112 weeks old. The initial rates of incorporation were 320 cpm/mg/min in the young animal and 145 cpm/mg/min in the old animal. Thus, the initial rate of incorporation in the old animals is 55% less than that of the young.

In order to further examine the ability of isolated mitochondria to synthesize protein, animals were pre-treated *in vivo* with cycloheximide to inhibit cytoplasmic protein synthesis and thereby remove the cytoplasmic contribution of mitochondrial proteins (Ibrahim *et al.*, 1979). As shown in Table 2, the young animals responded to this pre-treatment with a two-fold increase in mitochondrial protein synthesis. In contrast, the old animals studied had a markedly blunted response. In these experiments control incorporation was 5937 ± 209 cpm/mg and 3111 ± 284 cpm/mg in the young and old animals respectively.

Mitochondria isolated from old and young animals were incubated with the inhibitors of mitochondrial protein synthesis, α,α-dipyridyl (2×10^{-4}M) (Marcus *et al.*, 1980) and ethanol (0.15 M). This concentration of ethanol, while above the lethal dose in man, was chosen because it gave significant and reproducible inhibition of mitochondrial protein synthesis. Lower doses (0.1 M) showed variable effects inhibiting only about 50% of the mitochondrial preparations. As shown in Table 3, α,α-dipyridyl inhibited young mitochondria by 25% and ethanol by 21.9%. In the old animals the effects of α,α-dipyridyl and ethanol appeared additive with age; the old rats' mitochondria were 41.2% inhibited, while α,α-dipyridyl and ethanol increased this inhibition to 65.1% and 67.1% respectively, as compared to the young control.

In order to investigate if the effect of these inhibitors was due to a greater susceptibility of the old rats' mitochondria to these agents the data were expressed as percent of the young control and percent of the old control (Table 4). The percent inhibition was significantly greater for both α,α-dipyridyl and ethanol in the old rats' mitochondria, 48.8% and 48.0% respectively, as compared to 25.0% and 21.9% in the young.

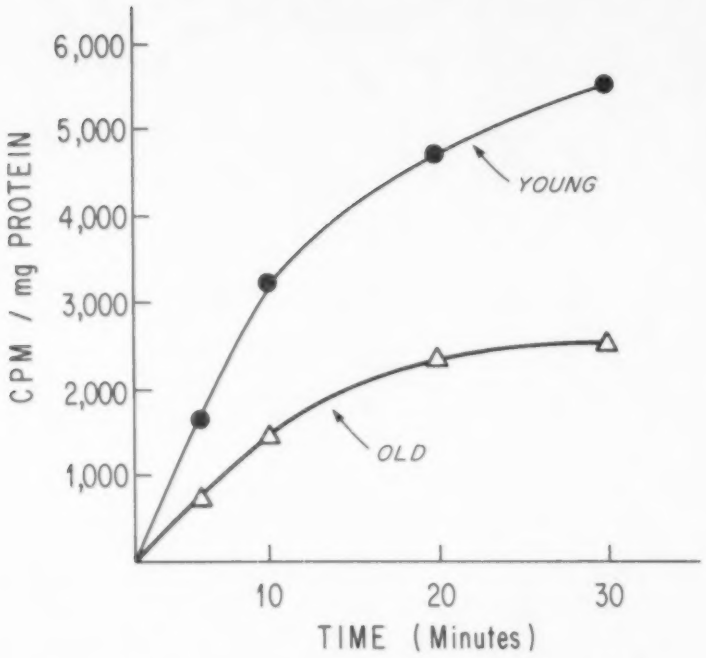

FIG. 1. Rate of Incorporation of L-(U-^{14}C) Leucine into Protein of Isolate Mitochondria of Young and Old Rat Livers. Incubations at 37°C were as described in Experimental Procedures. Samples were withdrawn at the time shown. This is a representative experiment performed in triplicate with less than 10% variation between duplicates. A small zero time was subtracted from each value. The young animal was 8 weeks old and the old animal was 112 weeks.

TABLE 2. EFFECTS OF *IN VIVO* ADMINISTRATION OF CYCLOHEXIMIDE ON *IN VITRO* MITOCHONDRIAL PROTEIN SYNTHESIS[a].

Percent of Control ± SEM[b]	
Young (2–3 months; n = 11)	Old (26–30 months; n = 3)
197 ± 13.8	116 ± 7.7[c]

[a]Rats were injected i.p. with cycloheximide, 10 mg/kg body weight, 2 hours before sacrifice. The mitochondria were isolated and incubated as described in Materials and Methods in triplicate with less than 10% variation between samples.

[b]Results are expressed as a ratio of control. Each control was a litter-mate not pre-treated with cycloheximide.

[c]Ratios of old statistically differ from ratios of young by Student *t*-test analysis of unmatched samples ($p < 0.00015$).

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TABLE 3. ADDITIVE EFFECTS OF AGE, α,α-DIPYRIDYL AND ETHANOL ON ISOLATED RAT LIVER MITOCHONDRIAL PROTEIN SYNTHESIS (N = 8)[a].

	Percent of Young Control					
	Young (2–3 months)			Old (26–30 months)		
	No Addition (Control)	α,α-Dipyridyl (2×10^{-4}M)	Ethanol (0.15M)	No Addition (Control)	α,α-Dipyridyl (2×10^{-4}M)	Ethanol (0.15M)
Mean	100	75.0	78.1	58.8	34.9	32.9
SEM	–	3.07	2.11	3.39	3.95	3.90
p value (differs from young control of 100% by Student *t*-test analysis)	–	<0.001	<0.001	<0.001	<0.001	<0.001

[a]A young control of 100% represents 6,687 cpm L-(U-^{14}C) leucine incorporated/mg protein/30 minutes at 37°C.

DISCUSSION

The data presented here shows that isolated mitochondria from the livers of old rats synthesize mitochondrial proteins at a markedly reduced rate as compared to those from young rats. The decrease in mitochondrial protein synthesis in the aged animal is due to neither a smaller amino acid pool size nor an increase in leakage of radiolabeled amino acid from the mitochondria of the aged rat. In addition the difference in initial rates indicate that we are measuring a basic physiologic difference in the aged rat. In order to determine if this age-related decrease in mitochondrial protein synthesis reflects an inhibition of cytoplasmic protein synthesis (Moldave *et al.*, 1979), *in vivo* cytoplasmic protein synthesis was inhibited with cycloheximide prior to studying *in vitro* mitochondrial protein synthesis.

We have confirmed that *in vivo* mitochondrial protein synthesis parallels cytoplasmic protein synthesis in rat liver (Ibrahim and Beattie, 1976). When the animal is treated with cycloheximide and *in vivo* mitochondrial protein synthesis is studied at two hours, there is an inhibition (Ch'ih, 1978). In the present study, however, we are investigating *in vitro* incorporation of leucine into mitochondria isolated from cells where cytoplasmic and mitochondrial protein synthesis were inhibited *in vivo*.

TABLE 4. COMPARATIVE EFFECTS OF α,α-DIPYRIDYL AND ETHANOL ON YOUNG AND OLD RAT LIVER MITOCHONDRIAL PROTEIN SYNTHESIS (n = 8).

	Percent Inhibition ± SEM	
Age of Rat	α,α-Dipyridyl (2×10^{-4}M)	Ethanol (0.15M)
Young (2–3 months)	25.0 ± 3.07	21.9 ± 2.11
Old (26–30 months)	48.8 ± 4.08[a]	48.0 ± 6.09[b]

[a]Percent inhibition of the old rats' mitochondrial protein synthesis by α,α-dipyridyl significantly differs from that of the young by Student *t*-test analysis ($p < 0.005$).

[b]Percent inhibition of the old rats' mitochondrial protein synthesis by ethanol significantly differs from that of the young by Student *t*-test analysis ($p < 0.005$).

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Our *in vitro* incubations following *in vivo* cycloheximide treatment demonstrate that only mitochondria from young animals are able to synthesize significantly greater amounts of mitochondrial proteins under these conditions. Ch'ih *et al.* (1978) using *in vivo* techniques for labeling mitochondrial protein, have shown a marked stimulation of isotope incorporation 24 hours after cycloheximide treatment, and a decrease after two hours. Our data suggests that *in vitro* incubation of isolated young rat liver mitochondria two hours after cycloheximide treatment allows the organelles to recover from the inhibitory effect of the cycloheximide induced decrease in cytoplasmic protein synthesis. These young mitochondria are then able to synthesize greater amounts of mitochondrial proteins, having been released from cytoplasmic control. In contrast, this ability is markedly diminished in the liver mitochondria from the old animals. It is not yet clear if this represents an inherent age-related decline in the ability of mitochondria to synthesize protein or if it reflects changes in cytoplasmic control mechanisms. What is clear, however, is that the decline in mitochondrial protein synthesis with age is not merely a simple reflection of a decline in cytoplasmic protein synthesis.

When mitochondria were exposed to inhibitors of mitochondrial protein synthesis, α-α-dipyridyl and ethanol, those from old animals showed greater inhibition. It appears, therefore, the old mitochondria are more susceptible to inhibition from these agents than the young. Thus, not only are mitochondria from old rats unable to synthesize mitochondrial proteins at the same rate as young rat mitochondria but they are more likely to be inhibited by exogenous agents and cannot respond to stimulation as well.

Our findings may also be of importance in understanding why δ-aminolevulinic acid synthase, the rate-limiting enzyme of heme synthesis (Granick, 1966) is produced in the cytoplasm under nuclear DNA control (Beattie and Stuchell, 1970; Hyashi *et al.*, 1969; Rosenberg and Marcus, 1974). For maximal cellular activity of this enzyme, it is transported to the mitochondrion and binds to a protein of the inner mitochondrial membrane which is synthesized on mitochondrial ribosomes (Beattie and Stuchell, 1970; Hyashi *et al.*, 1969; Rosenberg and Marcus, 1974) under mitochondrial DNA control (Mason and Schatz, 1973). Thus, it is possible the decrease with age in mitochondrial protein synthesis compounded with the inability to be stimulated and the increased susceptibility to inhibitors that we report here are responsible for the decrease in cellular δ-aminolevulinic acid synthase reported previously (Paterniti *et al.*, 1978).

We have recently shown that in spite of this decrease in mitochondrial protein synthesis in the liver from old rats, the cytochrome P_{450} content and microsomal heme concentration can be maintained under basal conditions (Ibrahim *et al.*, 1981). The variability in cytochrome P_{450} levels reported with advancing age in humans and animals (Kato and Takanaka, 1968; Vestal, 1978; Vestal *et al.*, 1975) might result from a variety of factors such as disease and inducers and inhibitors acting either directly on cytochrome P_{450} or acting independently on mitochondrial protein synthesis thereby decreasing heme synthesis. Thus, the older person or animal may be less capable of compensating for an increased demand of cytochrome P_{450} because of an overall decrease in mitochondrial protein synthesis (Ibrahim *et al.*, 1981) heme synthetic enzyme activity (Paterniti *et al.*, 1978) and cytoplasmic protein synthesis (Moldave *et al.*, 1979). This sequence of events might explain the decrease in liver microsomal enzyme induction reported with age (Adelman, 1971). Furthermore. A decrease in cytochrome P_{450} activity would explain the decreased ability of the elderly to metabolize drugs (Vestal, 1978) and possibly carcinogens.

Our results in the present study also suggest that the elderly may be more susceptible to the effects of iron deficiency, as shown by the α,α-dipyridyl experiments and ethanol.

SUMMARY

Mitochondria isolated from the livers of old rats (26–30 months) were found to incorporate 41% less leucine into mitochondrial proteins as compared to those from young rats (2–3 months). The initial rates of incorporation of label were 145 cpm/mg/minute for the "old" animals, and 320 cpm/mg/minute for the young animal. No difference in either amino acid pool size or leakage of label through the mitochondrial membrane was detected in the two age groups. Young rats were treated *in vivo* with cycloheximide (10 mg/kg) followed by isolation and incubation of their mitochondria *in vitro* 2 hours later. There was a two-fold increase in incorporation of leucine into mitochondrial proteins. In contrast, mitochondria isolated from old rats showed a markedly blunted response to cycloheximide pre-treatment. When mitochondria isolated from young and old rats were exposed to inhibitors of mitochondrial protein synthesis, α,α-dipyridyl (2×10^{-4}M) and ethanol (0.15M), the old mitochondria showed greater susceptibility to inhibition. These results suggest that the control of the biosynthesis of mitochondrial proteins is altered in the old animals.

Acknowledgements—This research was supported by USPHS Grant # AG 01399 from the National Institute of Aging, and USPHS Grant # AM 13532 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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Experimental Gerontology, Vol. 17, pp. 437–443, 1982
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PLASMA AND PITUITARY CONCENTRATIONS OF LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE AND PROLACTIN IN AGED, OVARIECTOMIZED CD-1 AND C57BL/6 MICE

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(*Received* 26 *January* 1982)

Abstract—Plasma and pituitary concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (Prl) were determined by ratioimmunoassay in young (2–4 months-old) and aged CD-1 (14–18 months-old) and C57BL/6 (16–22 months-old) mice one month after ovariectomy. In young, ovariectomized mice, plasma and pituitary concentrations of LH and FSH were significantly higher, whereas concentrations of Prl were significantly lower than those in control mice (sham-operated). In contrast, plasma concentrations of LH, FSH and Prl were not statistically different in aged, ovariectomized mice and aged, control mice. There were also no differences in pituitary concentrations of the three hormones when comparing the same aged C57BL/6 mice, although the aged, ovariectomized CD-1 mice exhibited higher pituitary levels of each hormone than those of their controls. The pituitary of the aged mouse responds differently to ovariectomy than that of the young mouse because of age-related changes in the ovary and/or hypothalamic-hypophyseal complex.

INTRODUCTION

AGE-RELATED changes in the hypothalamic-hypophyseal-ovarian complex are in part responsible for the loss of reproductive capacity in laboratory rodents. Radioimmunoassay studies conducted in our laboratory on aged oestrous and constant dioestrous C57BL/6 mice (Parkening *et al.*, 1980a) and on anoestrous Syrian hamsters (Parkening *et al.*, 1982) have shown that with advancing age a significant increase occurs in the circulating plasma concentrations of gonadotrophins and a decrease occurs in the plasma concentrations of prolactin (Prl). It is not known if these hormonal changes result from a dysfunctioning ovary or an increasing insensitivity of the pituitary-hypothalamic complex to gonadal feedback. In aged, ovariectomized, anoestrous hamsters, an insensitivity of the pituitary-hypothalamic complex appeared to be the primary reason for age-related changes in the secretion of pituitary hormones (Parkening *et al.*, 1982). To better determine what influence the ovary has on the pituitary-hypothalamic complex in aged mice, plasma and pituitary concentrations of gonadotrophins and Prl were measured in bilaterally ovariectomized mice.

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MATERIALS AND METHODS

Aged CD-1 Swiss albino (14–18 months-old) and C57BL/6 (16–22 months-old) mice and younger mature females (2–4 months-old) of both strains were obtained from Charles River Laboratories, Wilmington, Mass. The aged CD-1 females were acquired at 6 weeks of age and bred continuously until used in the experiment. The aged C57BL/6 mice were virgin females acquired from the NIH colony maintained by Charles River Laboratories. The latter females ranged from 15–20 months-of-age and were used in the experiment within 6–8 weeks of their delivery date.

The mice were housed under uniform temperature and lighting conditions (14 h light/10 h darkness) and provided with water and mouse chow *ad libitum*. Young mature and aged females of both strains were either bilaterally ovariectomized or sham-operated. One month following surgery, the ovariectomized mice were bled unanesthetized between 1000–1200 h by cardiac puncture using a heparinized syringe. Control females were checked after one month and bled as above on the day they exhibited a dioestrous vaginal smear.

All of the younger sham-operated mice exhibited a 4-day oestrous cycle, and most of the aged, sham-operated mice an irregular oestrous cycle or a constant leukocytic vaginal smear (only C57BL/6 mice showed the latter). None of the smears of the aged mice showed more than 2 days of cornified cells.

After bleeding, the pituitary gland was removed, weighed and cut in half. One-half was fixed for future immunocytochemical studies and the other half was reweighed and homogenized in 0.5 ml of 0.01 M phosphate-buffered saline (pH 7.6) containing 0.05 M EDTA and 0.1% sodium azide. The blood plasma and supernatant of the pituitary homogenates were stored at −85°C to permit an analysis of all samples in a given assay.

Procedures for the radioimmunoassay (RIA) of LH, FSH and Prl have been presented in detail elsewhere (Parkening *et al.*, 1980a, b; Collins *et al.*, 1980). NIAMDD rat-LH and rat-FSH RIA kits and a NIAMDD mouse-Prl RIA kit were used in determining concentrations of LH, FSH and Prl. In 90% of the mice, sufficient plasma was obtained from each animal to assay for all three hormones (single 100–200 μl samples for LH and FSH and duplicate 50 μl samples for Prl). Pituitary hormonal concentrations were determined in duplicate (200 μl of a 1/100 dilution of the supernatant from the pituitary homogenate for LH, 200 μl of a 1/10 dilution for FSH and 200 μl of a 1/20 dilution for Prl). No interassay variances occurred because all samples for an individual hormone were analyzed in a single assay. All intraassay variances were less than 10%.

A comparison of mean hormonal concentrations was made using the Student's *t* test. Statistical significance was determined at the 0.05 probability level.

RESULTS

The concentrations of LH in the plasma of the young mature CD-1 and C57BL/6 mice that had been ovariectomized were at least twice as great as those found in the sham-operated controls (Table 1). In contrast, LH concentrations in aged, ovariectomized mice of both strains were similar to those in the aged controls. Aged, control C57BL/6 mice, however, were found to have plasma LH concentrations that were more than twice as great as those in young mature mice ($p < 0.001$). In the pituitary, the concentrations of LH were much higher for the ovariectomized mice (except aged C57BL/6 mice) than for their controls (Table 1).

The concentrations of FSH in the plasma of young CD-1 and C57BL/6 mice that had been ovariectomized were at least 5 times as great as those in their controls; however, no differences were found between the FSH concentrations in aged, ovariectomized mice and aged, control mice (Table 1). The aged, sham-operated mice, therefore, had significantly higher concentrations of circulating FSH than the sham-operated, younger mice ($p < 0.001$). In contrast, young, ovariectomized mice had significantly higher concentrations of FSH than aged, ovariectomized mice ($p < 0.001$). Greater concentrations of FSH were found in the pituitary of the young, ovariectomized mice ($p < 0.001$) than in their controls, and in the aged, ovariectomized CD-1 mice ($p < 0.01$) than in their aged controls (Table 1). No differences existed between concentrations of FSH in aged, control and aged, ovariectomized C57BL/6 mice.

The concentrations of Prl that were found in the plasma of ovariectomized, young mice were considerably lower than those in the young controls ($p < 0.001$), whereas no differences were found in the concentrations of Prl in the aged, ovariectomized and aged,

TABLE 1. PLASMA AND PITUITARY LEVELS OF LH, FSH AND PRL IN YOUNG MATURE AND AGED OVARIECTOMIZED CD-1 AND C57BL/6 MICE.

	CD-1 Mice		C57BL/6 Mice	
	2–4 months-old	14–18 months-old	2–4 months-old	16–22 months-old
Plasma (ng/ml ± S.E.M.)				
LH				
Sham-operated	26.0 ± 0.8 (16)[a]	30.7 ± 2.1 (10)	24.2 ± 1.1 (17)	55.9 ± 5.8[h] (14)
Ovariectomized	54.3 ± 3.4[d] (20)	35.0 ± 3.5[j] (10)	66.3 ± 3.7[d] (22)	51.0 ± 2.7[i] (11)
FSH				
Sham-operated	284.7 ± 16.6 (16)	1102.3 ± 168.2[h] (9)	373.8 ± 16.9 (15)	973.5 ± 63.4[h] (15)
Ovariectomized	1545.2 ± 76.5[d] (20)	939.2 ± 93.5[k] (10)	1939.6 ± 88.1[d] (17)	1135.3 ± 84.4[k] (11)
Prl				
Sham-operated	75.4 ± 5.5 (16)	61.3 ± 4.3 (9)	62.4 ± 4.8 (14)	71.0 ± 3.2 (15)
Ovariectomized	36.5 ± 3.2[d] (20)	70.5 ± 5.0[k] (10)	33.6 ± 3.3[d] (17)	68.5 ± 5.4[k] (11)
Pituitary				
LH (ng/μg ± S.E.M.)				
Sham-operated	4.6 ± 0.4 (16)	5.2 ± 0.7 (13)	4.4 ± 0.3 (18)	5.4 ± 0.6 (15)
Ovariectomized	9.4 ± 0.5[c] (20)	8.2 ± 1.0[f] (10)	6.5 ± 0.4[d] (22)	6.9 ± 0.6 (11)
FSH (ng/μg ± S.E.M.)				
Sham-operated	3.2 ± 0.3 (16)	5.6 ± 0.5[h] (13)	3.7 ± 0.2 (18)	4.8 ± 0.4[g] (15)
Ovariectomized	15.0 ± 0.9[d] (20)	7.9 ± 0.5[f,k] (10)	9.9 ± 0.5[d] (22)	5.5 ± 0.3[k] (11)
Prl (ng/mg ± S.E.M.)				
Sham-operated	597.5 ± 24.1 (16)	373.9 ± 34.5[h] (13)	778.9 ± 44.4 (18)	532.0 ± 37.2[h] (15)
Ovariectomized	510.0 ± 20.0[b] (20)	467.0 ± 22.2[e] (10)	281.4 ± 23.6[d] (22)	495.5 ± 29.8[k] (11)

[a]Numbers of females assayed are in parentheses.

$p < 0.05$[b], $p < 0.01$[c], $p < 0.001$[d]: significantly different when comparing younger mature sham-operated and ovariectomized mice.

$p < 0.05$[e], $p < 0.01$[f]: significantly different when comparing aged sham-operated and ovariectomized mice.

$p < 0.05$[g], $p < 0.001$[h]: significantly different when comparing younger mature and aged sham-operated mice.

$p < 0.05$[i], $p < 0.01$[j], $p < 0.001$[k]: significantly different when comparing younger mature and aged ovariectomized mice.

control mice. Aged, ovariectomized and aged, control mice had concentrations of Prl in their plasma that were similar to those in the younger, control mice (Table 1). Concentrations of pituitary Prl were lower in ovariectomized, young CD-1 ($p < 0.05$) and C57BL/6 ($p < 0.001$) mice than in their controls. Aged, ovariectomized CD-1 mice had higher pituitary concentrations of Prl than their aged controls ($p < 0.05$), but no differences were discovered between pituitary Prl concentrations in aged, ovariectomized and aged, con-

trol C57BL/6 mice. The aged, control mice of both strains had lower concentrations of pituitary Prl than the young controls ($p < 0.001$).

DISCUSSION

A number of radioimmunoassay studies have shown that plasma concentrations of LH and FSH rise following gonadectomy in rats (Howland and Preiss, 1975; Gosden and Bancroft, 1976; McPherson *et al.*, 1977) and mice (Kovacic and Parlow, 1972). Gosden *et al.* (1978) have reported, however, that levels of serum LH in 3–6 months-old CBA mice, one month after ovariectomy, were similar to those in sham-operated controls. In contrast, Kovacic and Parlow (1972) showed that mice ovariectomized for 50 days had serum levels of LH that were significantly greater (approximately 2½ times higher) than those found in intact mice during dioestrus. In the present study, both strains of young mature mice had concentrations of LH that were approximately twice those found in sham-operated control, dioestrous animals.

The results that were obtained by Gosden *et al.*, (1978) may have been influenced by the strain of mouse they used. The CBA mouse is precocious in its loss of follicles and it is not known what causes such a loss. An early onset of acyclicity in this strain has been reported (Jones and Krohn, 1961) which implies premature hormonal changes.

Serum concentrations of Prl in C3H/St mice that had been ovariectomized for 2 weeks have been shown to be lower than those in sham-operated controls (Sinha *et al.*, 1972), although the difference was not significant. Pituitary concentrations of Prl in these same mice, however, were significantly less than those in ovariectomized animals. The discrepancies between that study and the present investigation in which significantly lower plasma Prl concentrations were found in younger ovariectomized CD-1 and C57BL/6 mice when compared to intact females, may result from the longer period the animals were ovariectomized (1 month) prior to bleeding or from strain differences. Female C57BL/St mice were shown to possess three times more serum Prl than C3H/St mice, but significantly less pituitary Prl (Sinha *et al.*, 1972).

The present study indicates that the hypophyseal-hypothalamic complex of the aged female mouse has little capacity to respond to ovariectomy. The response that does exist, as reflected by the concentrations of pituitary hormones that were found in 14–18 months-old CD-1 mice, apparently declines even further with increasing age, since no statistical differences were found in concentrations of pituitary hormones in ovariectomized and control 16–22 months-old C57BL/6 mice.

The pattern of hormonal secretion in young mature, ovariectomized mice in the present study was similar to that reported for young mature, ovariectomized rats (Huang *et al.*, 1976). Plasma concentrations of LH, FSH and Prl in aged CD-1 and C57BL/6 mice, ovariectomized for one month, remained similar to those in aged sham-operated mice. In contrast, aged rats (22–24 months-of-age) that either were in contrast oestrus or repetitive pseudopregnancy before ovariectomy, exhibited a rise in the levels of serum LH, FSH and Prl when compared with those levels found in younger, intact rats (Huang *et al.*, 1976). Moreover, even ovariectomized anoestrous rats (26–30 months-of-age) had significantly higher levels of FSH and Prl than intact, aged anoestrous rats (Huang *et al.*, 1976).

The pituitary of aged, anoestrous Syrian hamsters is also capable of responding to ovariectomy by increasing LH and FSH concentrations, although not to the same degree as the pituitary of younger, ovariectomized hamsters (Parkening *et al.*, 1982). While the

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hypothalamic-hypophyseal complex of anoestrous rats and hamsters appear to retain some ability to respond to the absence of negative feedback, the hypothalamic-hypophyseal complex of the aged mouse does not. The possibility exists, however, based on the elevated concentrations of LH in aged intact C57BL/6 mice and the higher concentrations of FSH in aged intact mice of both strains, that a poorly functioning ovary in these animals has already created a hormonal environment similar to that found in a gonadectomized mouse. Aged mice given steroid injections should provide answers as to whether the hypothalamic-hypophyseal complex is still capable of responding. In just such a study, aged C57BL/6J mice given exogenous oestradiol did not produce an LH surge comparable to that of young or middle-aged mice (Finch *et al.*, 1980). Younger mice in this same study exhibited a considerably higher LH surge than middle-aged mice, implying that the hypothalamus is responsible to a greater degree for changes in gonadotrophin secretion than are the ovarian steroids.

Aged rats and mice do not respond to exogenous GnRH in the same manner as younger animals. Serum concentrations of LH that were measured after an initial injection of GnRH were uniformly lower in aged rats of both sexes in comparison to those in younger rats (Miller and Riegle, 1978). The pituitaries of 16–22 months-old female C57BL/6 mice were also found to be considerably less sensitive than those of 2–4 months-old mice (Collins *et al.*, 1981). In another study, medial basal hypothalamic concentrations of GnRH in repetitively pseudopregnant 8–12 months-old rats (ovariectomized for 3 weeks) dropped appreciably from those of an intact group (Wise and Ratner, 1980). In the intact group, however, concentrations of GnRH were lower than those of younger rats with regular oestrous cycles. From this data it was concluded that the anterior pituitary was largely responsible for such changes in the older rats. In contrast, hypothalamic concentrations of GnRH in intact and ovariectomized rats exhibiting constant oestrus (8–12 months-old) were not statistically different from one another (although both were lower than those in intact, younger rats with regular oestrous cycles) and it was suggested that the major defect in these rats resided within the hypothalamus (Wise and Ratner, 1980). It is possible therefore, that the stage of irregular cyclicity exhibited by some older laboratory rodents is the direct result of age-related changes in either the anterior pituitary gland or the hypothalamus, rather than the entire complex.

In view of the many changes reported to be occurring in the concentrations of hypothalamic catecholamines, dopamine and serotonin in aged male and female rats (Clemens *et al.*, 1969; Quadri *et al.*, 1973; Meites *et al.*, 1979; Walker *et al.*, 1980) and aged male mice (Finch, 1973; Jonec and Finch, 1975); studies of a similar nature directed toward the hypothalamus of the older female mouse should add to our understanding of the age-related loss of reproductive capability by this species. Recent studies (Banerji *et al.*, 1982), conducted in this laboratory, on the levels of hypothalamic dopamine-β-hydroxylase (a norepinephrine-synthesizing enzyme), in four age groups of female C57BL/6 mice, have indicated that age-related differences occur in the levels of this enzyme during the evening of the proestrous LH surge. In mice 2–4, 7–8 and 12–13 months-of-age there was a significant increase of dopamine-β-hydroxylase activity at the same time there was a marked increase in circulating concentrations of LH. In contrast, 16–20 months-old mice which showed no appreciable increase in hypothalamic dopamine-β-hydroxylase activity from that on the morning of proestrus, contained less than half the concentrations found in 2–4 and 7–8 months-old mice. These data are consistent with studies (Meites *et al.*, 1979) done on the aging rat which suggested that age-related alterna-

tions in hypothalamic biogenic amines may be one of the primary causes of loss of reproductive capacity.

Since the pituitaries of aged mice do not respond to ovariectomy in the same manner as those of younger mice, the mechanisms for controlling the secretion of gonadotrophins and Prl may be less efficient in older mice. This deficiency may result from a reduction in hypothalamic and pituitary sensitivity to steroid feedback, changes in biogenic amine turnover, impairment in the release of GnRH or Prl-inhibiting factor (PIF), decreased binding of GnRH or PIF to pituitary cell receptors, or internal cellular alterations in the production and release of pituitary hormones. All of the above factors may be involved and research to elucidate the role each plays in the aging of the hypothalamic-pituitary complex is essential for understanding the causes of reproductive senescence.

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SUMMARY

Concentrations of LH, FSH, and prolactin (Prl) in the plasma and pituitaries of young (2–4 months-old) and aged CD-1 (14–18 months-old) and C57BL/6 (16–22 months-old) mice, were determined by radioimmunoassay, one month after ovariectomy. Plasma concentrations of LH and FSH in young, ovariectomized mice were 2–5 times greater than those in sham-operated control mice. Plasma concentrations of Prl in young, ovariectomized mice were significantly lower than those in young controls. Pituitary concentrations of LH and FSH were also statistically higher, whereas Prl concentrations were significantly lower in young, ovariectomized mice than in intact, control mice. In contrast, the plasma concentrations of LH, FSH, and Prl were not significantly different in aged, ovariectomized mice and aged, sham-operated mice. No differences existed between pituitary concentrations of LH, FSH, or Prl in aged, ovariectomized and aged, control C57BL/6 mice, although the pituitaries of aged, ovariectomized CD-1 mice had statistically higher levels of each hormone than those of their controls. Aged, sham-operated mice had significantly higher plasma concentrations of LH (C57BL/6 mice only) and FSH (both CD-1 and C57BL/6 mice) than young, control mice. No differences in plasma concentrations of Prl were found between young and aged, control mice. These data indicate that either the aged mouse ovary is no longer capable of producing adequate steroids to act on the hypothalamic-hypophyseal complex or (more probably) that the hypothalamic-hypophyseal complex in the aged mouse is less sensitive to gonadal feedback.

Acknowledgements—This research was supported in part by a NIH Grant (AG-00892) awarded to E.R. Smith.

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Experimental Gerontology, Vol. 17, pp. 445–452, 1982
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PHYSIOLOGICAL RESPONSES TO POSTURAL CHANGE IN YOUNG AND OLD HEALTHY INDIVIDUALS

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(*Received* 15 *February* 1982)

Abstract—The cardiovascular responses to 70° head-up tilt were compared in 9 healthy young, mean age 31 ± (S.D.) 3.2 years, and 8 healthy elderly subjects, mean age 71 ± 5.1 years. After 10 minutes tilt, qualitatively similar responses were found in the two groups. Quantitatively, however, heart rate, stroke volume, cardiac output, and peripheral resistance showed smaller differences between the supine and tilt positions in the older group. Blood pressure and plasma volume changes were similar in both groups. There is an age-associated effect in the cardiovascular response to change in posture in healthy people that resembles that found in congestive cardiac failure.

INTRODUCTION

THE SYNDROME of postural hypotension increases in incidence with increasing age so that up to 20% of old people suffer from the syndrome (Rodstein and Zeman, 1957; Johnson *et al.*, 1965; Caird *et al.*, 1973). Numerous factors have been associated with its development or potentiation (Fine, 1969; Gross, 1970; Cox *et al.*, 1972; Johnson and Spalding, 1974; Wollner and Spalding, 1978). The observation that these factors do not precipitate the syndrome in young individuals suggests some age-related deficit in postural reflexes (Landowne *et al.*, 1955). While it is well recognised (Landowne *et al.*, 1955; Strandell, 1964; Astrand *et al.*, 1973; Gribbin *et al.*, 1971) that "cardiovascular ageing" potentially will diminish reflex efficiency and compensation to postural stress in elderly people, quantitative data are scarce. There have been numerous studies of cardiovascular responses to posture change in young and middle-aged healthy individuals (Currens, 1948; Rushmer, 1959; Gauer and Thron, 1965; Lewis and Christianson, 1978; Matalon and Farhi, 1979; Mengesha and Bell, 1979) and in elderly patients with the syndrome of postural hypotension (Johnson *et al.*, 1965; Caird *et al.*, 1973; Fine, 1969; Gross, 1970; Cox *et al.*, 1972; Wollner and Spalding, 1978); however, there are few reports covering healthy, elderly subjects (Norris *et al.*, 1953; Granath *et al.*, 1965; Lee *et al.*, 1966).

The limited studies of healthy, elderly people have produced conflicting accounts. The results obtained have been variable and conflicting as a result of different methodologies

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and selection criteria. This prompted us to study the cardiovascular responses to posture change in a well-screened group of healthy, elderly subjects, and to compare their responses with those obtained in young healthy individuals, assessed under the same conditions using identical methods and apparatus.

METHODS AND PROCEDURE

Subjects

There were seventeen subjects (4 female) available for study. Nine (2F) were young – mean (± 1 S.D.) age 31 ± 3.2 years – and eight (2F) older individuals – mean age 71 ± 5.1 years. All were physically active and clinically healthy at the time of study. Persons with past or present history suggestive of cardiovascular, renal or neurological deficit, or, who were taking any drugs thought likely to interfere with postural reflexes, were excluded. All gave informed consent to the procedure after explanation, and the project was approved by the Hospital Ethical Committee.

Procedure

Subjects were studied in three positions on an electrically operated tilt-bed with foot support. After 20 minutes supine the head of the tilt-bed was elevated to 70° for 10 minutes, then lowered to supine again for a further 10 minutes. Measurements were obtained throughout at five minute intervals. For the purposes of this report results obtained immediately prior to change of position, i.e. at 15–20 minutes, 25–30 minutes and 35–40 minutes were recorded.

Stroke volume changes were measured from a continuously recorded impedance cardiogram, using a modified technique based upon the protocol of Kubicek et al. (1974). Details of the technique based on the present apparatus with a discussion of measurement errors and validity have been presented elsewhere (Lye and Vargas, 1981). Heart rate (beats/min) was determined from R-R measurements on the electrocardiogram. Cardiac output (L/min) was derived as the product of stroke volume and heart rate. Blood pressures (mmHg) were measured indirectly by standard sphygmomanometry. Systolic pressures were recorded as Phase I, confirmed by palpation, and diastolic levels were recorded as Phase V Korotkoff sounds. Mean blood pressure was computed as the diastolic level plus one-third of the pulse pressure (Berne and Levy, 1972). Peripheral vascular resistance was calculated from mean blood pressure and cardiac index (cardiac output corrected for body surface area). The result was converted to dynes. cm.$^{-5}$ sec. using the relationship of Apéria (1940). Blood samples were obtained with minimum stasis at the end of each tilt period. Changes in haematocrit were used to estimate plasma volume changes (Van Beaumont, 1972).

Statistical methods

Data were accumulated on a Nova 840 computer (Data General Co.). Differences between young and old subjects were assessed by the Student's "t" test with appropriate adjustment for unequal variances. Changes within groups were assessed by the paired "t" test. Significance was arbitrarily set at 5% or less (Bailey, 1959).

RESULTS

The healthy, young group showed a significant increase in heart rate and a significant decrease in stroke volume during the head-up position, Table 1. These changes resulted in a significant decrease in cardiac output which was compensated by an increase in peripheral resistance so that blood pressure changed little. The calculated change in plasma volume was −12.4%. All variables had returned to initial levels within 10 minutes of resuming the supine position. In the healthy, elderly group the same variables changed in the same direction as they did in the young group, Table 2, but the actual differences were smaller. The plasma volume of the elderly subjects decreased on average by 11.3%. Ten minutes after resumption of the supine position, only the blood pressures had returned to initial levels in the elderly group.

The average resting supine stroke volume of the young group was 35 ml/beat higher than the older subjects' result ($t = 3.72$; $p = .002$). The average initial supine differences

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TABLE 1. HAEMODYNAMIC RESULTS BEFORE, DURING AND AFTER HEAD-UP TILT
HEALTHY YOUNG SUBJECTS MEAN ± 1 S.D.

Variable	Supine A 15–20 min.	Head-up B 25–30 min.	Supine C 35–40 min.	Difference* A–B t/p	Difference* A–C t/p
Heart rate (beats/min.)	65.5 ±9.3	83.5 ±9.0	63.8 ±9.1	18.00 p < .001	1.51 p = .150
Stroke volume (ml/beat)	114.8 ±16.0	62.5 ±16.8	110.3 ±19.6	12.25 p < .001	1.25 p = .229
Cardiac output (l/min)	7.45 ±1.22	5.11 ±1.00	6.91 ±0.82	6.41 p < .001	2.01 p = .062
Peripheral resist. (dynes. cm.$^{-5}$ sec.)	27232. ±6615.	42609. ±8577.	28727. ±4672.	7.31 p < .001	1.80 p = .091
Systolic B.P. (mmHg)	107.2 ±9.7	106.8 ±9.9	104.5 ±9.7	0.26 p = .798	1.74 p = .101
Diastolic B.P. (mmHg)	69.3 ±3.8	78.5 ±5.1	70.1 ±1.6	7.69 p < .001	1.31 p = .209

*paired "t" test

in heart rate (t = 1.97; p = 0.068) and cardiac output (t = 1.95; p = .070) were not significantly different between the two groups. The average peripheral vascular resistance supine was much higher in the older subjects (t = 3.05; p = .008) as were resting systolic (t = 5.36; p = .001) and diastolic (t = 3.62; p = .005) blood pressures. Inspection of Tables 1 and 2 shows that inter-individual variation is higher in the older group compared with the younger group. This applies to all variables recorded. The largest difference in variability expressed as coefficient of variation is in resting stroke volume—14.0 and 28.3% young and old respectively. The smallest difference in variability occurs in systolic blood pressure—9.1 and 11.2% in the same two groups.

TABLE 2. HAEMODYNAMIC RESULTS BEFORE, DURING AND AFTER HEAD-UP TILT
HEALTHY ELDERLY SUBJECTS MEAN ± 1 S.D.

Variable	Supine A 15–20 min.	Head-up B 25–30 min.	Supine C 35–40 min.	Difference* A–B t/p	Difference* A–C t/p
Heart rate (beats/ml)	80.1 ±17.1	89.7 ±14.9	76.7 ±15.8	6.80 p < .001	2.85 p = .013
Stroke volume (ml/beat)	79.7 ±22.6	62.4 ±13.8	72.7 ±18.7	4.41 p < .001	2.83 p = .013
Cardiac output (L/min)	6.18 ±1.46	5.58 ±1.36	5.41 ±1.15	2.06 p = .058	4.65 p < .001
Peripheral resist. (dynes. cm.$^{-5}$ sec.)	42000. ±12746.	50183. ±16601.	48160. ±14268.	2.81 p = .014	4.53 p < .001
Systolic B.P. (mmHg)	140.9 ±15.8	139.4 ±15.7	142.2 ±16.7	2.08 p = .056	0.75 p = .466
Diastolic B.P. (mmHg)	81.4 ±8.7	92.2 ±9.3	82.5 ±8.9	13.73 p < .001	1.38 p = .189

*paired "t" test

Head-up tilt produces qualitatively similar responses in both the young and the old group (Fig. 1). There are, however, marked quantitative differences (Table 3). Thus, the increase in heart rate of the young group is significantly less than the average response in the old group. The young decrease stroke volume and cardiac output and increase

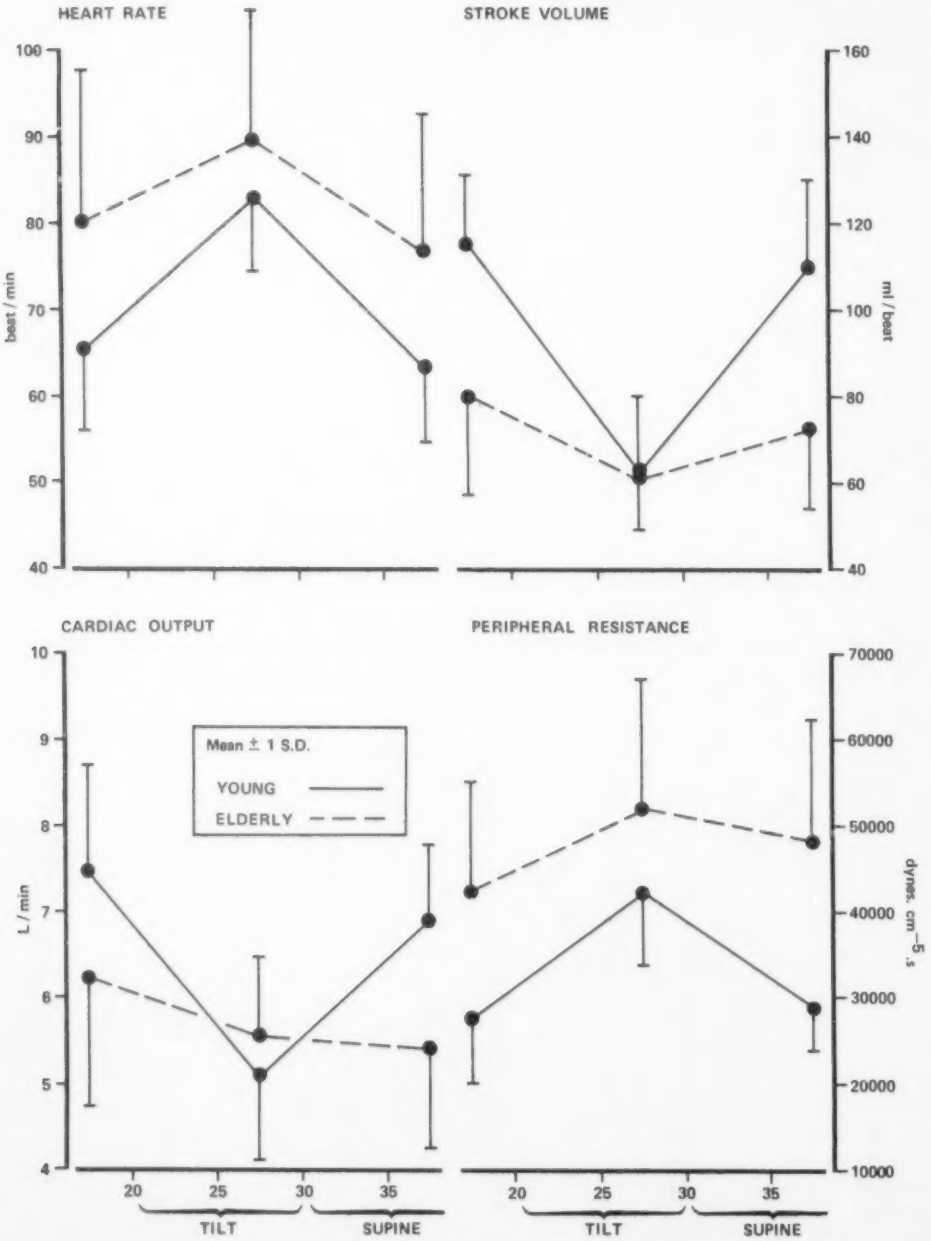

FIG. 1. The effects of tilt on selected variables in young and old healthy individuals.

TABLE 3. DIFFERENCES IN RESPONSES TO HEAD-UP TILT IN YOUNG AND ELDERLY SUBJECTS.

	Young Subjects	Elderly Subjects	Differences	
			t	p
Heart rate	28.6 ± 10.2	13.1 ± 8.4	3.40	.004
Stroke volume	−45.5 ± 14.0	−19.3 ± 15.5	3.66	.002
Cardiac output	−29.8 ± 19.9	−8.5 ± 19.7	2.21	.043
Peripheral resist.	61.5 ± 33.1	21.5 ± 27.0	2.71	.016
Systolic B.P.	−0.2 ± 5.6	−1.4 ± 1.6	0.59	.564
Diastolic B.P.	13.9 ± 7.7	13.2 ± 4.0	0.21	.836
Plasma volume	−12.5 ± 1.5	−13.1 ± 2.0	0.27	.791

Mean (% change from supine) ± 1 S.D.

peripheral resistance much more than the elderly group. The changes in systolic and diastolic blood pressures are the same in both groups.

DISCUSSION

The present results demonstrate clear quantitative differences in cardiovascular variables both at rest supine and during 70° head-up tilt between healthy young and healthy old individuals. The qualitative changes in the variables of the two groups were all in the same direction. In essence the older individuals show an attenuated response to head-up tilt whilst maintaining a suitable blood pressure for cerebral perfusion. The young group demonstrates the excesses of youth in their exaggerated responses, while achieving the same objective.

An early study (Norris et al., 1953) of 140 males, age range 20–92 years, showed that heart rate and blood pressures in young and old change in the same direction and essentially to the same degree. Unfortunately, these individuals were not selected nor screened to confirm cardiovascular health; neither are the numbers of individuals in each age group indicated. A later, more detailed study from Sweden (Granath et al., 1964) reported that there were no significant age differences in heart rate changes though both stroke volume and cardiac output decreased more in the young group than in the older group on going from a supine to a sitting position. Unfortunately, these results are not directly comparable with other data obtained by passive tilting because venous return is increased by the leg muscle contraction in the more active manoeuvre. A final study (Lee et al., 1966) on the effects of aging on posture change was unable to demonstrate any quantitative difference between eight men aged more than 47 years compared with the responses obtained in seven men aged less than 34 years during 45° head-up tilt. In this latter study, the overall age range was too small to demonstrate differences and further 45° head-up tilt is probably insufficient to provide an adequate cardiovascular stress (Matalon and Farhi, 1979).

The present results, showing attenuation of cardiovascular reflexes to posture stress in

healthy elderly subjects, are reminiscent of similar changes brought about by cardiac failure (Sanchez-Zambrano and Spodick, 1974; Abelman, 1976), in which there is a high sympathetic adrenergic output with increased resting pulse rate and peripheral resistance associated with an increased plasma volume. Careful investigation of the present subjects revealed no past or present features of cardiac failure. Perhaps, further, more invasive methods of investigation of carefully screened, selected and healthy elderly people are required before those physiological changes which increase liability to postural hypotension are understood.

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SUMMARY

While many elderly patients suffer from an inability to maintain blood pressure on changing from the supine to the head-up position (postural hypotension), little is known of the effects of aging per se upon cardiovascular response to change in posture. The present study compares cardiovascular responses in young (mean-age 31 years) and elderly (mean age 71 years) healthy individuals. The results show that while the pattern and direction of change in cardiovascular variables are the same in the two groups there are marked and significant quantitative differences. The differences are particularly marked in relation to heart rate and stroke volume responses. Plasma volume changes were the same in the two groups. Blood pressures (systolic, diastolic and mean) in both groups did not change significantly on head-up tilt. The response of the healthy elderly subjects is similar to what is expected in cardiac failure, suggesting an age-related attenuation of cardiac reserve, and hence a potential liability to develop postural hypotension with increasing age.

Acknowledgements—John Cooley gave excellent technical support and Brian Faragher provided statistical advice. The Universidad de Guanajuato and CONACYT (Mexico) gave financial support to Dr. Enrique Vargas and the Needham Trust enabled the purchase of equipment. Without the young and elderly volunteers no study would have been possible.

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Experimental Gerontology, Vol. 17, pp. 453–461, 1982
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A LONGITUDINAL METABOLIC PROFILE OF SWISS ALBINO MICE DURING AGING

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(*Received* 15 *February* 1982)

Abstract—The consequence of aging on carbohydrate and lipid metabolism of the Swiss albino mouse was examined in a complete aging profile. The profile was developed by obtaining groups of at least 10 weanling mice at 2–3 month intervals for approximately a 2-year period. Body weight plateaued at approximately 10 months-of-age. Maximal heart and liver weights were found in 3-month-old mice. Plasma glucose concentration was highest in 1-month-old mice, declined rapidly during the first 3 months-of-age and then gradually throughout the profile. Liver glycogen content spiked sharply in 2-month-old mice, decreasing sharply to a level maintained in mature animals. A second peak in hepatic glycogen occurred in 15-month-old mice with a precipitous decrease taking place in older animals. Plasma concentrations of glycerol, 3-hydroxybutyrate and triacylglycerols, indices of lipid metabolism, were remarkably constant and showed no consistent trend with age. The level of triacylglycerols in the heart peaked in 2-month-old mice and then decreased gradually in older animals. The maximal content of triacylglycerols in the liver was found in 10- to 20-month-old mice. Hepatic levels of triacylglycerols decreased precipitously in senescent Swiss albino mice.

INTRODUCTION

AGE-RELATED CHANGES in lipid metabolism have been documented in both man and rat (Kritchevsky, 1980; Tobey *et al.*, 1981). There is considerable evidence that the concentration of triacylglycerols in plasma or serum increases with age in both species (Carlson *et al.*, 1968; Reaven, 1978; Tobey *et al.*, 1981). This age-related rise in the concentration of triacylglycerols in plasma, at least in the rat, has been attributed to an increase in very low density lipoprotein-triacylglycerols (VLDL-TG) secretion proportionate to an associated increase in liver weight, accompanied by a relative decline in efficiency of VLDL-TG removal from plasma (Tobey *et al.*, 1981). The cause of this age-related decrease in removal of VLDL-TG could be due to the aging process itself yet the degree of obesity may also have an influence.

Disposal of a glucose load is also progressively impaired during aging (Davidson, 1979). The mechanism of this aging effect on carbohydrate metabolism remains obscure and may indeed be heterogenous in nature. A decrease in insulin effectiveness or sensitivity may indeed be a primary cause of the aging effect on carbohydrate metabolism (Davidson, 1979). Age-dependent changes in carbohydrate and lipid metabolism could in fact be interrelated. Accumulation of triacylglycerols in tissues of older animals may be linked to the decreased insulin sensitivity during aging.

In the present study, a longitudinal metabolic profile of the Swiss albino mouse during aging was developed. Parameters of both carbohydrate and lipid metabolism as well as growth were monitored from immediately post-weaning until senescence.

MATERIALS AND METHODS

Materials

Pentobarbital sodium was obtained from Sigma Chemical Company, St. Louis, Missouri. The enzymes and co-factors necessary for the analysis of plasma and tissue constituents were obtained either from Sigma, Boehringer Mannheim Corporation, Indianapolis, Indiana or P-L Biochemicals, Milwaukee, Wisconsin.

Mice

Male Swiss albino [ARS HA (ICR) f] were obtained as weanlings from ARS/Sprague-Dawley, Madison, Wisconsin. A complete aging profile was developed by obtaining groups of at least ten weanling mice at approximately 4–8 week intervals over a two year period. They were kept in groups of 5 mice per cage in shoebox-type mouse cages. The ambient temperature was maintained at 21 ± 1°C. All mice were maintained on a 12-h light-dark cycle (lights on from 0600–1800 h) with *ad libitum* access to Lab Chow (Ralston Purina Co., St. Louis, Missouri) and tap water. The aging profile, consisting of eleven groups of 3 to 10 mice each ranging in age from 1 to 24 months, was terminated on 2 mornings (0800–1200 h), separated by only 1 day, in a manner selected to minimize intra- and interday variability. Several mice, 22 and 24 months in age respectively, were excluded at the time of the experiment due to the presence of overt pathology, e.g. hepatic nodules and various tumors.

Mice were killed under pentobarbital anesthesia (110 mg/kg, intraperitoneal) by cardiac puncture. Heart, and then liver were sampled by freeze-clamp technique at the temperature of liquid nitrogen (Wollenberger *et al.*, 1960) and were subsequently stored at this temperature until assayed. Lightly heparinized plasma separated from blood obtained by cardiac puncture was stored at −15°C.

Analytical methods

Plasma glucose was determined enzymatically without prior protein precipitation (Bergmeyer *et al.*, 1974). Lactate (Hohorst, 1965), alanine (Williamson, 1974), glycerol (Wieland, 1974), and 3-hydroxybutyrate (Williamson and Mellanby, 1965), in neutralized perchloric acid extracts of plasma, were determined fluorometrically by enzymatic methods.

The glycogen content of liver samples was determined enzymatically (Huijing, 1970; Hung and Menahan, 1980) and the results were expressed in terms of glucose equivalents from glycogen. The concentration of triacylglycerols in homogenates of liver in water was determined by the method of Laurell (1966). Plasma samples or homogenates of heart in water were extracted and saponified as described by Laurell (1966) but the triacylglycerol-glycerol was quantitated fluorometrically by an enzymatic method (Wieland, 1974).

RESULTS

The influence of aging on body and liver weight of male Swiss albino mice is shown in Figs. 1a-c. Body weight plateaued at approximately 10 months-of-age and remained relatively stable throughout the rest of the aging profile (Fig. 1a). Maximal liver weight was attained in 3-month-old mice (Fig. 1b). However, the relative contribution of liver weight to body weight was highest at 1 month-of-age, the earliest age studied (Fig. 1c). As a result of the rapid increase in body weight (Fig. 1a), the relative liver weight declined rapidly until a constant value was achieved at 10 months-of-age. Similar to liver weight (Fig. 1b), absolute heart weight increased rapidly in mice from 1 to 3 months-of-age (Fig. 2a). A gradual increase in heart weight occurred throughout the aging profile so that the decrease in relative heart weight in 10- to 15-month-old animals was blunted (Fig. 2b) in comparison to markedly lower relative liver weights found in similarly aged mice (Fig. 1c). In older mice (>20 months), the relative contribution of heart weight to body weight increased to a value seen in mice younger than 3 months-of-age (Fig. 2b).

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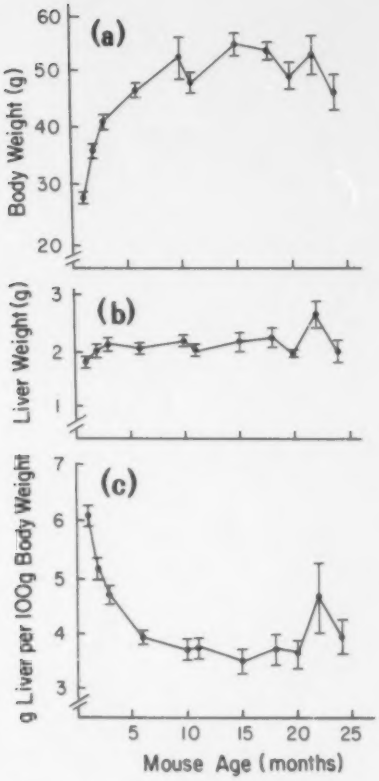

FIG. 1. The influence of mouse age on body and liver weight. Each point represents the mean ± S.E. for 3 to 10 mice per group. (a) Body weight and (b) liver weight as a function of age. (c) The relative contribution of liver weight to total body weight at different ages.

Age-related changes in carbohydrate metabolism were monitored by quantitating certain plasma and liver constituents in mice from immediately post-weaning to senescence. Plasma glucose concentration was highest in 1-month-old mice, the earliest age examined (Fig. 3a). A rapid decrease in plasma glucose was found in mice between 1 and 3 months-in-age with a more gradual decline in concentration occurring throughout the rest of the aging profile. Concentration of lactate in plasma, an index of Cori-cycle activity, decreased in mice up to 3 months-of-age (Fig. 3b), paralleling changes in plasma glucose in similarly-aged mice (Fig. 3a). After remaining relatively unchanged in mature mice (5–15 months), plasma lactate values increased in older mice (Fig. 3b). The plasma concentration of alanine, an important precursor for gluconeogenesis, was remarkably similar in mice of all age groups (Fig. 3c). Liver glycogen content spiked in 2-month-old mice with a rapid decline to a level that remained constant in mature mice (Fig. 4). A rapid increase in

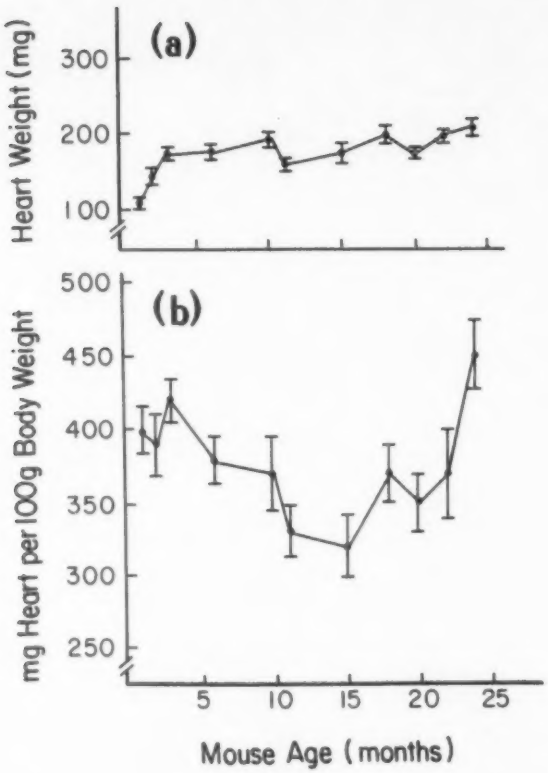

FIG. 2. Heart weight as a function of mouse age. Each point represents the mean ± S.E. for 3 to 10 mice per group. (a) Total heart weight and (b) the relative contribution of heart weight to body weight at different ages.

hepatic glycogen content was found in mice between 11- and 15-months-of-age with a precipitous decline then taking place in older mice.

Certain plasma constituents were selected as indices for evaluating age-related changes in lipid metabolism. Concentration of glycerol, 3-hydroxybutyrate, and triacylglycerols in plasma was notably similar in mice at all ages (Figs. 5a-c). Even though the concentration of triacylglycerols in plasma remained relatively constant throughout the aging profile (Fig. 5c), neutral fat content of both liver and heart was very dependent on the age of the mouse (Fig. 6a, b). From an initially low value in young mice, hepatic triacylglycerol content increased markedly in mice between 6 and 10 months-of-age, remaining relatively stable in mature mice until a precipitous decrease occurred in senescence (Fig. 6a). On the other hand, the concentration of triacylglycerols in the myocardium was highest in 2-month-old mice and decreased throughout the remainder of the aging profile (Fig. 6b). The level of triacylglycerols in the heart of a 24-month-old mouse was less than one-half that found in young animals (<5 months old).

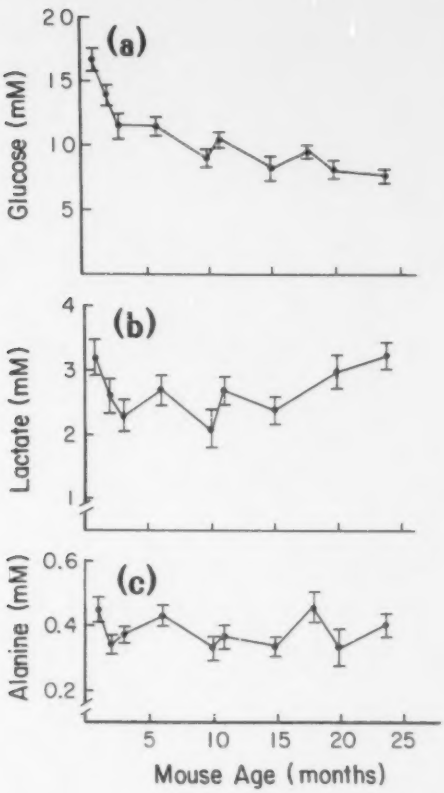

FIG. 3. Concentration of glucose, lactate and alanine in blood plasma as a function of mouse age. Each point represents the mean ± S.E. for 4 to 9 mice per group. Plasma (a) glucose, (b) lactate and (c) alanine concentrations in mice of different ages.

DISCUSSION

Though the cellular changes responsible for decline in normal immune functions with age have been elucidated in the mouse (Makinodan and Peterson, 1962; Makinodan, 1978), age-related changes in both carbohydrate and lipid metabolism in this species have not been examined to any significant extent. Since most of the previous studies with experimental animals have been restricted not only to the rat but also to lipid metabolism (Kritchevsky, 1980), a longitudinal metabolic profile of the Swiss albino mouse during aging was developed in the present study. The body weight of the male Swiss albino mouse fed *ad libitum* plateaued at approximately 10 months-of-age. Yet, the rat with the possible exception of the Fisher 344 strain gains weight throughout its life-span (Story *et al.*, 1976; Talesara and Mohini, 1978). Age-related changes in the relative contribution of liver or heart weight to total body weight in Swiss albino mice during the period of rapid growth

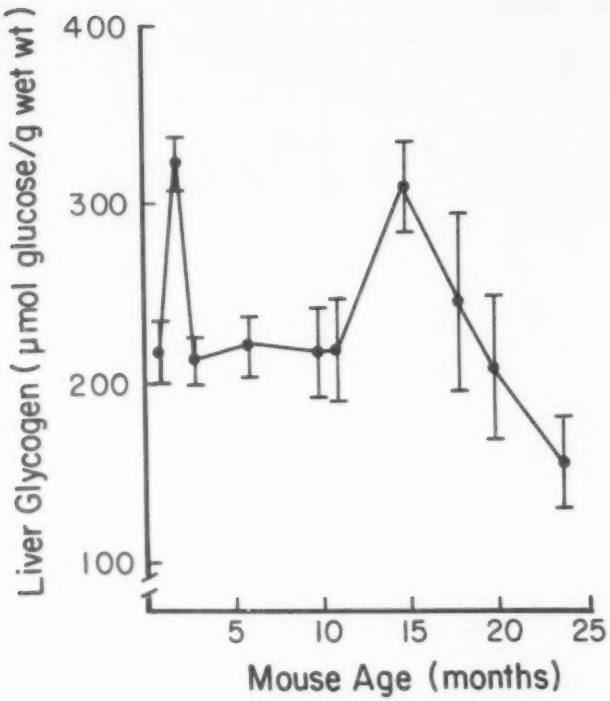

FIG. 4. Hepatic glycogen content for a longitudinal aging profile in the mouse. Each point represents the mean ± S.E. for 4 to 10 mice per group.

immediately post-weaning till 10 months-of-age are similar to those reported for Holtzman rats (Talesara and Mohini, 1978).

Most of the age-related changes in carbohydrate and lipid metabolism of the Swiss albino mouse took place during at least 1 of 4 time intervals: namely the first 3 months-of-age when rapid development occurred; the period during which maximal body weight was achieved (3 to 10 months-of-age); a plateau phase from 10 to 20 months-of-age; and finally senescence. Immediately post-weaning, plasma glucose concentration was highest in Swiss albino mice declining rapidly during the first 3 months-of-age and then slowly through the remainder of the aging profile. A precipitous decrease in blood glucose has also been reported to occur in the Sprague-Dawley rat between 1 and 4 months-of-age (Carlson *et al.*, 1968). In mice between 1 and 3 months-of-age, the concentration of lactate in plasma decreased in parallel to that of glucose. In older mice (>15 months), plasma lactate levels gradually increased while glucose concentration was still slowly declining. A sharp peak in hepatic glycogen content occurred in mice at 2 months-of-age with a decline to a level in 3-month-old mice that was maintained until maximal body weight was achieved at 10 months-of-age. The concentration of glycogen in the liver then increased rapidly in 15-month-old Swiss albino mice and subsequently declined continuously in aged animals.

With regard to various indices of lipid metabolism determined in plasma, a remarkable lack of age-dependency in the Swiss albino mouse was seen. The sharp age-related in-

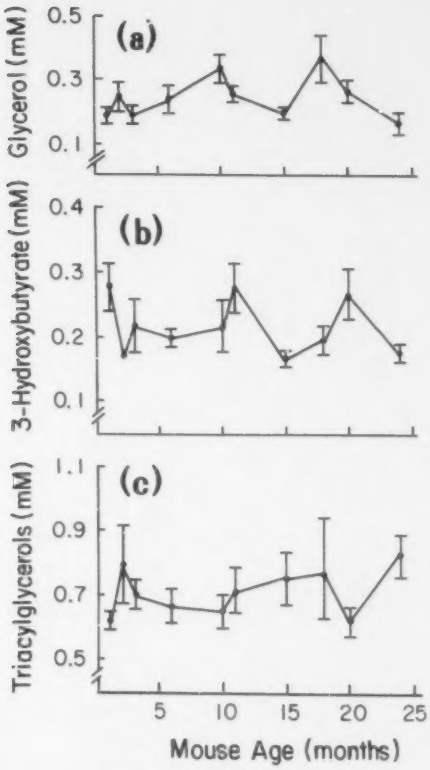

FIG. 5. Concentration of glycerol, 3-hydroxybutyrate and triacylglycerols in blood plasma as a function of mouse age. Each point represents the mean ± S.E. for 4 to 9 mice per group. Plasma (a) glycerol, (b) 3-hydroxybutyrate and (c) triacylglycerol concentrations in mice of different ages.

crease in plasma triacylglycerols found in the rat (Carlson *et al.*, 1968; Story *et al.*, 1976; Reaven, 1978; Uchida *et al.*, 1978; Tobey *et al.*, 1981) was not seen in Swiss albino mice in the present study. Similar to the rat (Carlson *et al.*, 1968), the concentration of glycerol in plasma showed no consistent age trend. Though the concentration of triacylglycerols in plasma remained relatively constant in Swiss albino mice throughout the aging profile, the level of neutral fat in both heart and liver did change in an age-dependent manner. The level of triacylglycerols in the heart peaked in 2-month-old mice during the period of maximal growth of the myocardium. Thereafter, a gradual decrease in the myocardial content of triacylglycerols was found in Swiss albino mice throughout the rest of the aging profile, a response similar to that reported for the rat heart (Carlson *et al.*, 1968).

The hepatic content of triacylglycerols in Swiss albino mice changed in a manner distinctly different from the other parameters determined in the present study. Maximal hepatic content of neutral fat was found in Swiss albino mice in the plateau phase (10 to

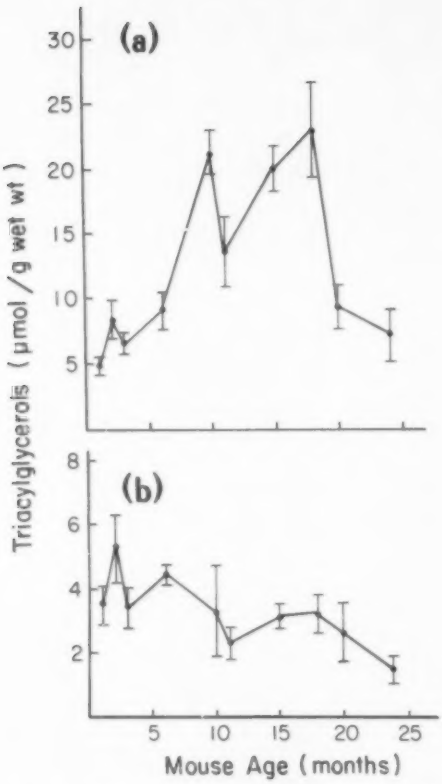

FIG. 6. Liver and heart triacylglycerol content as a function of mouse age. Each point represents the mean ± S.E. for 3 to 10 mice per group. Levels of triacylglycerols in (a) liver and (b) heart in mice of different ages.

20 months-of-age) after maximal body weight was attained. This is similar to the age-dependent changes reported for hepatic triacylglycerols in the rat (Story *et al.*, 1976; Egge and Murawski, 1978; Uchida *et al.*, 1978). The profile of the age-related changes in the concentration of hepatic triacylglycerols in the present study is almost identical to our previous findings on the influence of age on the triacylglycerol content of the mouse fat cell (Jolly *et al.*, 1980). The factors responsible for the precipitous decrease of triacylglycerol levels in both hepatocytes and adipocytes in senescent Swiss albino mice may involve an age-dependent loss of responsiveness or sensitivity to insulin even though hormone-stimulated glucose uptake is normal (Davidson, 1979). Though age-dependent changes in tissue content of triacylglycerols can be demonstrated, the Swiss albino mouse has a remarkably constant concentration of neutral lipid in plasma.

Acknowledgements—This work was supported in part by grants AM 07681 and HL 07564 from the National Institutes of Health; Milwaukee Divison, American Cancer Society; American Heart Association, Wisconsin Affiliate; American Diabetes Association, Wisconsin Affiliate; and TOPS Club, Inc., Obesity and Metabolic

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Research Program, Milwaukee, WI. The technical assistance of Mrs. L.-Y. Hung and Mr. W.T. Hron and the preparation of typescript by Mrs. L. Binder are gratefully acknowledged.

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Experimental Gerontology, Vol. 17, pp. 463–471, 1982
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THE EFFECT OF DIETARY RESTRICTION ON CELL DIVISION POTENTIAL, DNA CONTENT AND ENZYME LEVELS IN THE ROTIFER *ASPLANCHNA BRIGHTWELLI*

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(*Received* 3 *March* 1982)

Abstract—Dietary restriction in the rotifer *Asplanchna brightwelli*, carried out by lengthening the interval between feedings, causes an increase both in mean lifespan and in the length of the reproductive period. In the present study, we found that the rate of nuclear division in the gastric glands and vitellarium of the rotifer, as determined by daily nuclear counts, was retarded by dietary restriction. Spectro-fluorimetric measurements show that total DNA content remains constant from the beginning to the end of the lifespan, and is unaffected by dietary restriction. Lactic dehydrogenase and malic dehydrogenase activities were also measured throughout the lifespan of the rotifer, and were not affected by dietary restriction.

INTRODUCTION

THE PURPOSE of this paper is to explore the cytological and biochemical consequences of dietary restriction in the rotifer *Asplanchna brightwelli*. Dietary restriction can be imposed on this species of rotifer by decreasing the number of times at which they are fed paramecia: the control group of rotifers is fed every 12 hours while the group on dietary restriction is fed every 36 hours. The results of a previous study showed that both the mean lifespan and the length of the reproductive period were increased through this method of dietary restriction (Verdone-Smith and Enesco, 1982).

Rotifers have been used frequently in aging studies because they have a short lifespan which can be altered by manipulating environmental factors such as temperature and diet. From mammalian studies, it was proposed that dietary restriction acted by slowing down cell division (Lamb, 1977). Since rotifers are primarily eutelic, the only organs where cell division might be influenced by dietary restriction are those where cell division continues into adult life: the gastric glands which aid in digestion and the vitellarium which provides nutrients to the developing embryo (Jones and Gilbert, 1977).

The first experiment dealt with the effect of dietary restriction on the patterns of nuclear division in these two organs. Next, DNA content was measured throughout the lifespan of the rotifers to see whether total DNA concentration was affected by dietary

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restriction. Also, lactic dehydrogenase and malic dehydrogenase activities were measured throughout the lifespan of the rotifers. If dietary restriction acts to slow down metabolic activities, we might expect lower enzymatic activities in these two important enzymes of the energy cycle.

The effect of dietary restriction on longer lived rotifers (*Philodina*) has been previously examined by Fanestil and Barrows (1965). Events that took place over the 20-day lifespan of *Philodina* would be compressed into the 6-day lifespan of *Asplanchna*, so that trends might be accentuated in the short lived species.

The purpose of this study was thus to determine whether the control and dietary-restricted rotifers displayed differences throughout their lifespan regarding a) the rate of nuclear division in the gastric glands and vitellarium, b) total DNA content and c) levels of lactic dehydrogenase and malic dehydrogenase, as an index of protein synthetic ability or metabolic efficiency.

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MATERIALS AND PROCEDURES

Culture and experimental methods

The rotifer used in this study was *Asplanchna brightwelli* clone 4B61, obtained from Dr. John Gilbert, Dartmouth College, Hanover, NH. The rotifers were kept in a Cerophyll infusion medium innoculated with *E. coli* and *Paramecium caudatum* as described in a previous paper (Enesco and Verdone-Smith, 1980).

The rotifers were placed singly in the wells of tissue culture clusters #3524 (from Costar, Cambridge, Mass.). The chambers were checked every 12 hours for offspring which were then transferred to experimental chambers. Only newborn rotifers were used for experiments since their age could be estimated accurately.

All experimental cultures were kept in the dark at 18°C and determinations of death and offspring production were taken every 12 hours. The controls were fed every 12 hours while the group on the dietary-restriction was fed *Paramecia* every 36 hours. At the times when the rotifers were not fed, a medium containing Cerophyll only was used to replenish the wells.

Nuclear counts

The procedure for counting nuclei in vitellarium (yolk-gland) and gastric glands was based on a method described by Birky and Field (1966). Rotifers were removed from the two groups on the appropriate day and time (day 1 to day 6) and were rinsed in distilled water. They were then transferred to a petri dish containing 50% ethanol and fixed for 3 minutes. A few fixed rotifers were placed on a slide. After the excess ethanol was drained by tilting the slide, a coverslip was gently applied. Following this fixation, the organs and their nuclei were clearly visible and nuclei could be counted using a Zeiss Ultraphot II phase contrast microscope at a magnification of 200X. Twenty rotifers from both control and restricted groups were used for each of the daily nuclear counts.

DNA content

The method used for measuring DNA content was based on the method of Le Pecq and Paoletti (1967) where a dye, ethidium bromide (Sigma Chemicals Co., St. Louis, Missouri), was used to bind nucleic acids. The binding caused fluorescence that could be measured spectrofluorimetrically.

Twenty rotifers were collected from control and restricted groups on day 1 through day 6, and rinsed 3 times in deionized-distilled water. The rotifers were then placed in sonication vessels containing 1.0 ml of deionized-distilled water and sonicated in an ice bath for 30 seconds using a Cell Membrane Disruptor/Sonic 300 working at 35% capacity. So that only DNA content would be measured, ribonuclease-A was added to the sonicate at a concentration of 20 μg/ml for 1 hour at room temperature to break down RNA. The ethidium bromide solution was prepared at a concentration of 7.5 μg/ml in a 0.1M Tris-HCl/NaCl buffer solution, pH 7.5. A standard curve for DNA ranging from concentrations of 0.01 μg/ml to 5.0 μg/ml was also prepared using DNA sodium salt (reagent grade, Kodak Chemical Co., Rochester, N.Y.) in the above buffer solution. After 20.0 ml of the ethidium bromide solution was added to all the tubes, the samples were read on an Aminco/Bowman spectrofluorimeter at ex = 546 nm and em = 590 nm.

Enzyme activities

Levels of malic dehydrogenase and lactic dehydrogenase activities were determined spectrofluorimetrically by measuring amounts of fluorescent β-NAD released as described by Lowry, Roberts and Kapphahn (1957), by Fanestil and Barrows (1965), and by Verdone-Smith (1981).

Three rotifers from control and dietary restricted groups were collected at the appropriate age (day 1 to day 6) and were rinsed in deionized-distilled water. The rotifers were rinsed 3 times in sonication medium made up of 0.005 M Tris-Cl, pH 8.2 and 0.7 M NaCl in 0.05% bovine plasma albumin. The rotifers were then placed into sonication vessels containing 1.0 ml of the above buffer and sonicated for 30 seconds in an ice bath.

The incubation medium for both dehydrogenases was composed of 0.1 M Tris, 0.05% bovine protein albumin, 0.025 M nicotinamide and 0.001 M NADH. For the lactic dehydrogenase assay, 15 μl of sonicate were placed with 15 μl of incubation medium; the substrate used was 15 μl of 1×10^{-3}M sodium pyruvate, pH 7.4 and the tubes were incubated in a water bath at 37.5°C for 90 minutes. For the malic dehydrogenase assay, the procedure was the same except that the substrate used was 7.5×10^{-4}M neutralized oxalacetate, pH 8.8 and the reaction mixture was incubated for 45 minutes.

The standard curve for NAD had a range of 0.05 to 3.0 μg NAD/ml, and was prepared in sonication medium. Following incubation, all tubes were placed in an ice bath and 20 μl of 0.45N HCl were added to destroy residual NADH, followed by 10 μl of 6N NaOH; the tubes were then heated for 10 minutes in a 60°C water bath to develop fluorescence. After this time period, 2.0 ml of deionized-distilled water were added to all the tubes and the samples were read at ex = 360 nm and em = 460 nm.

Statistical Analysis

For the nuclear counts study, the *t*-test was used to determine whether the number of nuclei in control and restricted groups was statistically different, comparing counts on every day of life. A paired *t*-test was performed to find out whether DNA content and enzymatic activities were statistically different between the two groups throughout their 6-day lifespan.

RESULTS

Figure 1 shows the survival curves of rotifers fed every 12 hours and those fed every 36 hours. The mean lifespan of the control group was 5.44 ± .28 days while the mean lifespan of the group on dietary-restriction was 6.08 ± .34 days. Although the lengths of pre-reproductive phase were similar in both groups, the rotifers on dietary-restriction displayed a slightly longer reproductive phase which may be related to the slight increase in mean lifespan. The data on dietary restriction and reproduction has been presented in an earlier paper (Verdone-Smith & Enesco, 1982).

Table 1 shows the average number of nuclei present on each day in the gastric glands and vitellarium of control rotifers as compared to those on dietary restriction throughout their 6-day lifespan. Since two gastric glands were present in each rotifer, the average of the two was used. A *t*-test revealed that there were no significant differences in the number of nuclei in the two gastric glands from the same organism. The pattern of statistically significant differences in nuclei between control and experimental groups is analyzed in Table 1.

The pattern of nuclear counts obtained on each day is shown in Fig. 2. In control rotifers, the average number of nuclei in the vitellarium was at its lowest on day 1 and steadily increased until a peak was reached on day 5. On day 6, the average nuclear number decreased sharply. In the rotifers on dietary restriction, the average number of nuclei in the vitellarium was also low on day 1 and steadily increased until day 5 but at a significantly slower rate than in the controls. On day 6, the nuclear number decreased slightly in this group and a small sample of 7-day-old rotifers revealed that by this age, the nuclear number decreased even further.

In the gastric glands of the controls, the average number of nuclei increased from day 1

FIG. 1. The survival curve of *A. brightwelli* in a control group fed every 12 hours is compared with that of an experimental or restricted group fed every 36 hours. (T = 18°C, N = 24).

to day 4 and then slowly decreased to day 6. In the restricted group, the pattern was the same, but again the increase occurred at a significantly slower rate than in controls. On day 6, the nuclear number finally reached the average previously attained by control rotifers.

The pattern obtained in the only two dividing organs of the rotifers suggests that the rate of nuclear division was slowed down by dietary restriction.

The DNA content in control and restricted rotifers during the 6-day lifespan is shown in Fig. 3. DNA concentration was the same in both groups on day 1 and day 6. Changes in DNA content from days 2 to 5 were attributed to changes in reproductive rate and to the presence of offspring within the adult rotifers. Although the results indicated that DNA content was lower in restricted rotifers, a *t*-test revealed no significant differences between the 2 groups ($t = 2.47$, $p < .05$).

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TABLE 1. AVERAGE NUMBER OF NUCLEI IN THE VITELLARIUM AND GASTRIC GLANDS OF CONTROL AND DIETARY-RESTRICTED *Asplanchna brightwelli* ON EACH DAY OF LIFE. (N = 20)
(Note that there are 2 gastric glands/rotifer and that the average is given.)

	VITELLARIUM Average number of nuclei ±S.E.		GASTRIC GLANDS Average number of nuclei ±S.E.	
Age Inverval (days)	Control	Experimental	Control	Experimental
0–1	20.5 ± .57	19.6 ± .62	5.93 ± .15	5.50 ± .14
1–2	28.4 ± .63	26.4 ± .60*	6.70 ± .22	6.20 ± .18*
2–3	29.9 ± .70	27.2 ± .78*	9.80 ± .19	9.40 ± .25
3–4	28.6 ± .77	29.8 ± .79	10.70 ± .33	9.50 ± .28**
4–5	30.9 ± .98	31.3 ± .65	10.20 ± .27	9.40 ± .29**
5–6	24.8 ± .57	30.3 ± .90*	9.80 ± .28	10.30 ± .25
6–7	–	(26.5 ± 1.15)[1]	–	–

[1] = (N = 6).
Significant differences between control and experimental groups according to *t*-test.
* = $p < .05$
** = $p < .01$

The levels of lactic dehydrogenase activity measured by the release of fluorescent NAD for control and restricted rotifers are given in Fig. 4. In control rotifers, the level of lactic dehydrogenase activity increased slowly during the first 3 days of life followed by a sharp increase to day 4. After this time, the level of enzymatic activity increased slightly and leveled off by day 6. The same pattern was obtained in the group on dietary restriction except that the levels were slightly lower for the first 3 days of life; there was also a sharper increase from day 3 to day 4 and the activity levels kept increasing until day 6.

The levels of malic dehydrogenase activity for both groups of rotifers for 6 days are shown in Fig. 5. The patterns obtained for the 2 groups were identical to those of lactic dehydrogenase activity. A paired *t*-test on both sets of results revealed no significant differences in enzymatic activities between control and dietary-restricted rotifers throughout the 6-day lifespan ($t = 1.58, .553, p < .05$).

DISCUSSION

The reason why dietary restriction increases lifespan in numerous organisms is unknown. Some experiments suggest that factors which retarded growth and development may also postpone senescence (Comfort, 1979). Lamb (1977) proposed that dietary restriction may slow down the "biological clock" which governs the rate of aging.

In the present experiment, increasing the feeding interval from 12 to 36 hours in *A. brightwelli* increased the length of the reproductive phase and increased lifespan. Since rotifers are eutelic, it cannot be assumed that a "biological clock" based on the number of cell divisions is regulating the rate of the aging process in a non-dividing system. It could be suggested that rotifers that displayed a longer lifespan may have a reduced "rate of living" where certain events involved in reproduction and senescence occurred at a slower pace.

The results of the nuclear count experiment show that the pattern of nuclear division for the vitellarium and gastric gland is different in the restricted as compared to the con-

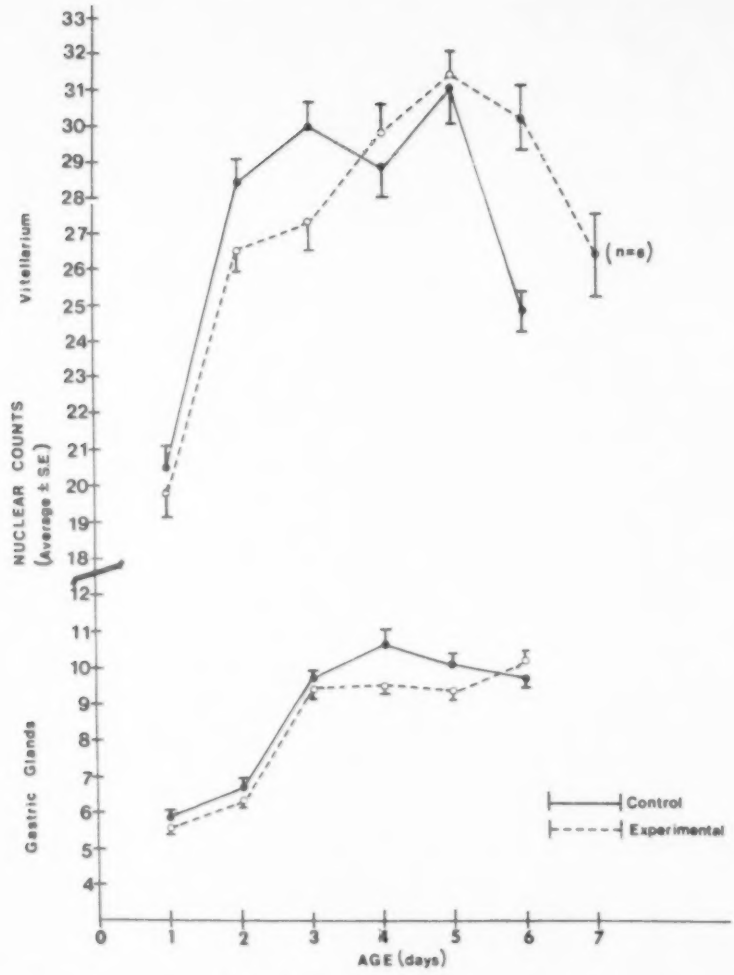

FIG. 2. The average number of nuclei in the vitellarium and gastric glands of control and experimental or restricted *A. brightwelli* are shown throughout their lifespan. The daily counts were made on 20 rotifers per group.

trol rotifers. Although the average nuclear number in the gastric glands gradually increased in both groups, the rate of increase was slower in the restricted group. In both groups, the nuclear number in the vitellarium increased until day 5 and then decreased in the post-reproductive period. Again, the rate of these events was slower in the restricted rotifers.

The data show that nuclear division in the gastric glands and vitellarium was slowed down through dietary restriction; this could be related to the delayed senescence and lengthened reproductive period in this group.

Spectrofluorimetric measurements of DNA content show that there were no significant differences between control and restricted rotifers throughout their lifespan. The results

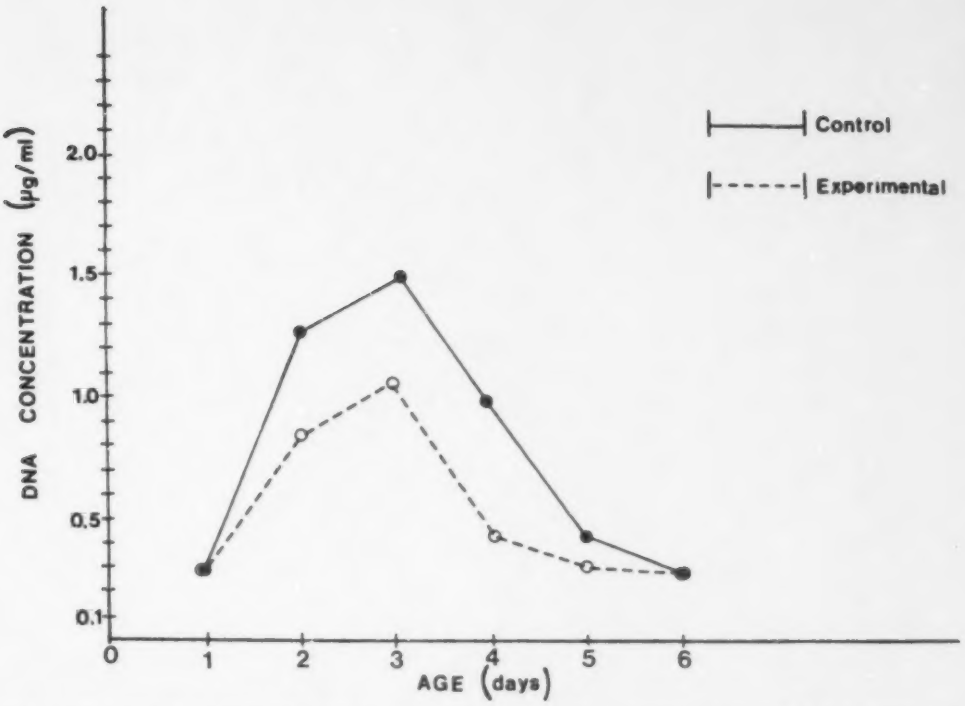

FIG. 3. DNA content on each day of life is compared for control and experimental or restricted *A. brightwelli*. Note that the initial and final values remain constant. (T = 18°C; 20 rotifers per group).

show identical DNA concentrations in both groups on day 1 and day 6; on these days, the probability of the presence of offspring was lower than on any other day; thus it can be assumed that there was no change in total DNA content with age in these rotifers. This observation confirms that rotifers are basically eutelic. The small increase in nuclear counts observed in the vitellarium and gastric glands were not of sufficient magnitude to influence the total DNA content of the organism.

Measurements of lactic dehydrogenase and malic dehydrogenase activity throughout the 6-day lifespan showed that there were no significant differences between control and restricted rotifers. Since the levels of activity of both enzymes increased during and after reproduction, there was no indication that a decline in enzymatic activity occurred with aging. Fanestil and Barrows (1965) have shown that levels of lactic dehydrogenase and malic dehydrogenase activity were markedly affected in underfed *Philodina* as compared to controls. In this rotifer species, levels of activity increased in early life, became relatively stable during adulthood and then decreased during senescence. The sequence of events was delayed in *Philodina* by underfeeding. *Philodina* have lifespans that are 4 to 5 times longer than *A. brightwelli*. Since death occurs one day after the end of the reproductive period in *A. brightwelli* (Verdone-Smith and Enesco, 1982), the "senescent" or post-reproductive period is extremely short in this species, and we may not be able to detect any gradual enzyme changes during post-reproductive senescence.

The various experiments reported here show that dietary restriction increased both the

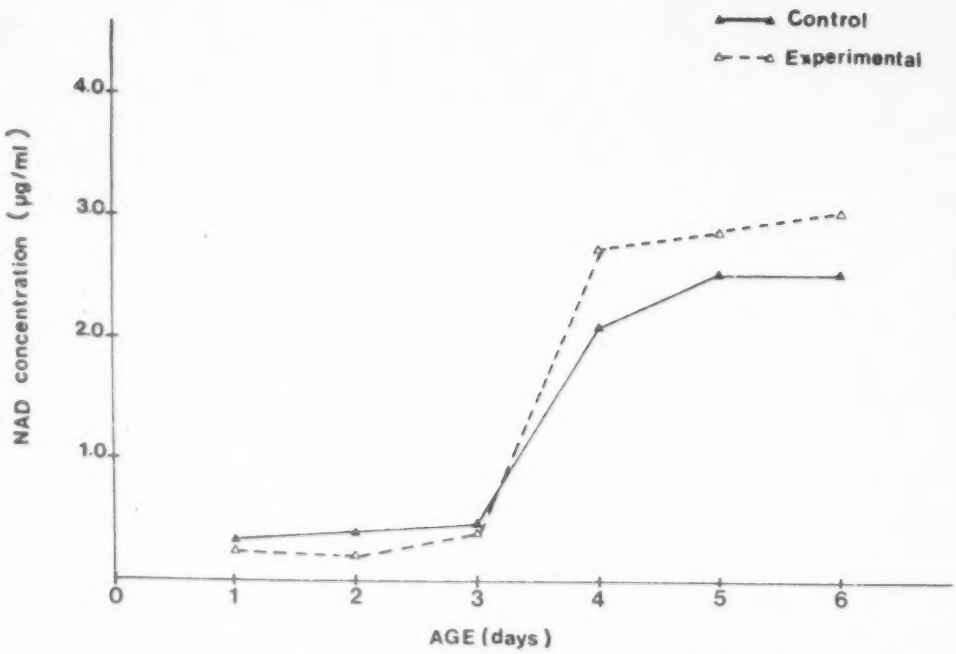

FIG. 4. This figure shows the pattern of lactic dehydrogenase activity, as determined by NAD releases, for control and experimental (dietary-restricted) *A. brightwelli* throughout lifespan. Note that the values increase at 3 days for both groups. (T = 18°C; 3 rotifers/group).

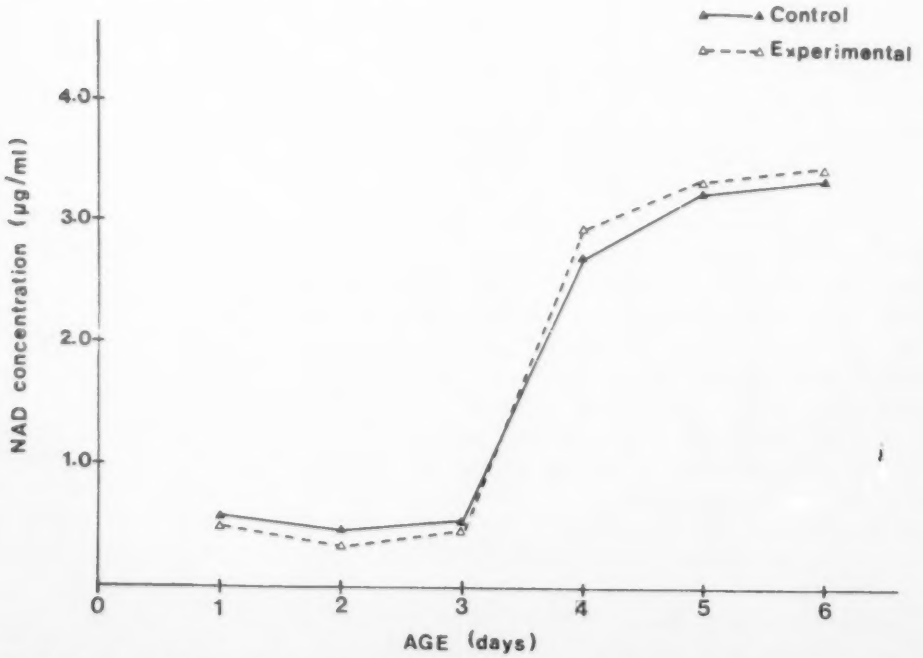

FIG. 5. The pattern of malic dehydrogenase activity, as determined by NAD release, is shown for control and experimental (dietary-restricted) *A. brightwelli* throughout a 6-day lifespan. (T = 18°C; 3 rotifers/group).

length of life and reproductive period in *A. brightwelli*; these increases may be correlated with slower rates of nuclear division as seen in the gastric glands and vitellarium of the rotifers. Dietary restriction did not affect DNA content or levels of lactic dehydrogenase and malic dehydrogenase activity throughout the lifespan of *A. brightwelli*.

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Experimental Gerontology, Vol. 17, pp. 473–480, 1982
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0531-5565/82/060473-08$03.00/0
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PROLONGATION OF S PHASE AND WHOLE CELL CYCLE IN WERNER'S SYNDROME FIBROBLASTS

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(*Received* 3 *March* 1982)

Abstract—The cell cycle was determined in early passage fibroblasts from Werner's syndrome and normal subjects. The average cell cycle time was prolonged in Werner's syndrome cells due to changes in the duration of S phase. Using alkaline sucrose gradient sedimentation, the rate of DNA elongation was examined, and no difference was observed between Werner's syndrome cells and normal cells.

INTRODUCTION

WERNER'S SYNDROME (WS), a rare autosomal recessive disorder (Rabbiosi and Borroni, 1975; Björneerg, 1976; Goto *et al.*, 1981; Salk *et al.*, 1981a), is clinically characterized by a distinctive habitus (short stature and slender extremities), a variety of feature of premature senescence (alopecia, arteriosclerosis, cataract, etc.), scleroderma-like skin change and endocrinological abnormalities (Epstein *et al.*, 1966; Goto *et al.*, 1978). This syndrome has been described as a caricature of aging (Epstein *et al.*, 1966) or segmental progeroid syndrome (Martin, 1978; Norwood *et al.*, 1979). In comparison with human diploid skin fibroblasts, examination with skin fibroblasts from WS patient may provide information about this genetic disease.

Normal human fibroblasts in culture have a limited life span (Hayflick, 1965; Martin *et al.*, 1970) and the change in cell cycle duration in late passage culture is primarily due to change in G1 phase (Yanishevsky *et al.*, 1974; Grove and Cristofalo, 1977). The decreases of labeling index (Cristofalo and Sharf, 1973; Schneider and Mitsui, 1976; Nichols *et al.*, 1977) and the rate of DNA elongation (Petes *et al.*, 1974) have been also reported. As compared with normal fibroblasts, striking diminution in life span (Martin *et al.*, 1970; Goldstein, 1979; Norwood, 1979; Salk *et al.*, 1981a), the decrease of cells in S phase (Tanaka *et al.*, 1979), the decrease of population growth rate (Salk *et al.*, 1981a)

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and increase of exponential growth rates (Salk *et al.*, 1981a) have been described in cultured fibroblasts from WS patients. In addition, slow rate of DNA replication is reported in some WS fibroblasts (Fujiwara *et al.*, 1977). The duration of the interphase periods of the cell cycle of WS cells, however, are not clearly examined yet. In this report, the cell cycle was analyzed in 3 normal fibroblasts derived from 3 subjects of various ages and 5 WS fibroblasts derived from 5 patients of various ages in early passage. The percentage of nuclei synthesizing DNA was also determined in these cells. Then, the rate of DNA chain elongation were examined in the same cells by alkaline sucrose gradient sedimentation, for explaining the abnormal cell cycle of WS cells. Possible causes of the different mode of replication in WS cells compared with normal cells will be discussed.

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METHODS AND PROCEDURE

Cell strains

We used cultured skin fibroblasts from five WS patients (Goto *et al.*, 1978; Matsumura *et al.*, 1980) and three normal subjects (Table 1). In this study, all cells were used in early passage. Donor ages were spread widely.

Cell cycle analysis

The duration of the interphase periods of the cell cycle was determined by the percent labeled mitosis (PLM) method of Quastler and Sherman (1959) with minor modifications. Cells were inoculated with 2 ml of MEM with 10% fetal calf serum (FCS, GIBCO) at a concentration of 1×10^4 cells/ml into 2-chambered tissue culture chamber slides (Lab-Tek Products). After incubation in 5% CO_2-incubator for 2 days, cultures in log phase growth were exposed to ^{3}H-thymidine at 0.5 μCi/ml (specific activity 25 Ci/mmol, Amersham) for 30 min. After being washed twice with MEM containing 10% FCS, cells were incubated with MEM containing 10% FCS continuously. All media were prewarmed at 37°C. Duplicate cultures were stopped at 1-h intervals over the next 11-h and then 2-h intervals for further 33-h. After being washed once with calcium, magnesium-free phosphate-buffered saline, cells were fixed with the mixture of acetate and ethanol (1:3) for 1 min. Air-dried slides were rinsed with 2% perchloric acid 4 times and with water, and then dipped in Sakura NR-M2 emulsion. Exposure time was 1–2 weeks. Autoradiographs were developed with Rendol and Renfix (Fuji Photo Film Co.). A minimum of 100 mitoses throughout the slides were scored for each sample. The PLM were plotted against the time. The duration of the interphase periods of the cell cycle was measured using the method of Macieira-Coelho (1973).

TABLE 1. CELLS.

Cell identification No.	Donor age	Sex	Final PDL†	PDL	Previously reported identification No.	
					Goto, et al. (1978)	Matsumura, et al. (1980)
N* 1	26	F	ND§	4.0		
N 2	40	M	ND	13.4		
N 3	86	M	ND	6.2		
WS** 1	19	M	6.7	2 5	1	1
WS 2	29	F	ND	3.0	4	2
WS 3	43	M	6.5	4.3	10	6
WS 4	60	M	9.8	2.7	15	5
WS 5	61	M	7.0	2.9		

*N: normal subject
**WS: Werner's syndrome
†PDL: population doubling level
§ND: not determined

Labeling Index

The percentage of nuclei synthesizing DNA (labeling index) for 30 min pulse period was determined by autoradiography. Samples were prepared as described above and about 1,000 cells were examined.

Alkaline sucrose gradient sedimentation

Alkaline sucrose gradient sedimentation was according to the method of Abelson and Thomas (1966) and Fujiwara *et al.* (1977) with some modifications. Cells shown in Table 1 (1×10^5 cells/∅ 35 mm dish) were incubated with MEM containing 10% FCS at 37°C for 2 days. After being washed once with MEM containing 10% dialized FCS, 2×10^{-6}M 5-fluorodeoxyuridine and Eagle's non-essential amino acids, cells were preincubated with the same medium at 37°C for 30 min to exhaust the endogenous thymidine nucleotide pools. Cells were then incubated with 5×10^{-6}M ^{3}H-thymidine (70 Ci/mmol, Amersham) in the same medium for 3 min. The medium was removed and the cells were washed twice with MEM containing 10% FCS and 5×10^{-3}M thymidine (growth medium). After being incubated with the growth medium for 10, 30 or 120 min, the cells were lysed with 0.25% sodium dodecylsulfate, 0.01M EDTA and 0.15M sodium bicarbonate, pH8.0 and digested with 1 mg/ml proteinase K (Boehringer-Mannheim) for 16-h at 37°C. After addition of 5N NaOH to make 0.3N, the lysate (0.2 ml of about 1.25×10^5 lysed cells/ml) was layered on top of 4.8 ml linear gradient from 5 to 20% (W/V) sucrose containing 0.8M NaCl, 0.2N NaOH and 0.01M EDTA, pH12.5. The gradients were centrifuged at 30,000 rpm for 2-h at 20°C in Hitachi RPS40-T2 rotor with ^{14}C-labeled T4D bacteriophage DNA (66×10^6 in the denatured form) as an internal reference. After the centrifugation, 5-drop fractions were collected onto Whatman type 3MM filter paper disc (∅ 24mm). Discs were washed with 5% trichloroacetic acid 3 times and 95% ethanol, dried and assayed for radioactivity. The average molecular weights (MW) between fractions 1 and 32 or 33 in the sedimentation profiles were calculated from the formula of Abelson and Thomas (1966). For control study, normal cells were labeled with 0.5 μCi/ml (25 Ci/mmol, Amersham) of ^{3}H-thymidine for 24-h and subjected to alkaline sucrose gradient sedimentation.

RESULTS

Cell cycle analysis

Fig. 1 shows the PLM curves for typical two cases: one is normal fibroblasts and the other is Werner's syndrome fibroblasts; at various time after pulse-labeling with ^{3}H-thymidine. Other samples from normal subjects and WS patients showed essentially the same curves, respectively. In all cases the first peak was the almost 100% level but the second peak showed lower level. No difference of the curve was found among the cells from variously aged donors, but the delay of the appearance of the second peak was found in WS cells. From the figures, the duration of interphase periods of the cell cycle was calculated (Macieira-Coelho, 1973) and the results are shown in Table 2. T_{G1} means the duration of G1 phase + (0.5 × mitotic phase) and T_{G2} means the duration of G2 phase + (0.5 × mitotic phase). T_S and T_C mean the duration of S phase and whole cell cycle, respectively. In normal cells, T_C was 18.8 to 19.0-h (mean = 18.9) and T_S was 7.4 to 8.9 (mean = 8.2). In early PDL, cells from variously aged donors did not show any difference of T_C, T_S, T_{G1}, T_{G2}. In WS cells, T_C was 20.4 to 22.4 (mean = 21.2 and T_S was 10.5 to 11.4 (mean = 10.9). Namely, cell cycle time of WS fibroblasts was slightly, but significantly elongated in comparison with the normal cells of early passage, mostly due to the prolongation of S phase ($p < 0.05$). No appreciable change in the G1 and G2 phase was observed.

Labeling index

The percentage of cells being able to incorporate ^{3}H-thymidine during 30 min labeling period is shown in Table 2. In normal cells, the labeling index was 32.0 to 37.8 (mean = 35.3) and the older the donor was, the lower the labeling index was (in equally early passages) (Schneider and Mitsui, 1976). On the other hand, the labeling index of WS cells

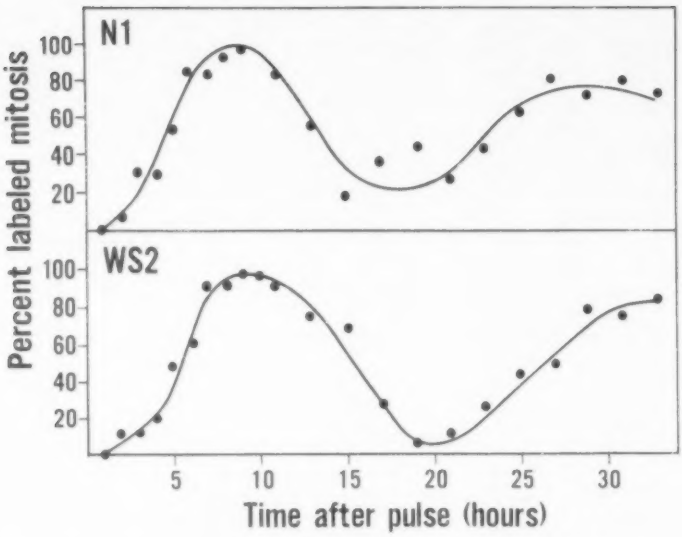

FIG. 1. Percent labeled mitosis (PLM) of normal and Werner's syndrome cells. Only typical cases are shown (N1 and WS2). From these kinds of figures, the duration of the interphase periods were calculated by the method of Macieira-Coelho (1973). PLM curves were generated by plotting the percentage of labeled mitosis in log phase culture after a 30 min pulse exposure to ^{3}H-thymidine. In details, methods are described in the text.

were 17.6 to 29.8 (mean = 25.0). No simple correlation between labeling index and donor age was found in this case.

Alkaline sucrose gradient sedimentation

In order to know about the cause of the prolongation of S phase in WS cells, we examined the changes in the sedimentation profiles of pulse-labeled DNA with chasing time. In Fig. 2, sedimentation profiles of labeled DNA from two typical cells, one is normal and the other is WS, are shown. The sedimentation velocity of newly synthesized DNA increased rapidly with chasing time from 0 to 120 min. No difference of the sedimentation profiles was observed among these cells and other normal and WS fibroblasts. Calculated mean molecular weights are plotted against the chasing time in Fig. 3. In our study, after chasing for 120 min, newly synthesized DNA in normal cells was almost the same size as the continuously labeled DNA (MW = 178×10^6). In WS cells, after 120 min chase, the newly synthesized DNA was the same size as in normal cells (bulk size). The MW of single-stranded DNA labeled for 3 min with no chase was not zero but 68 to 80×10^6 not only in normal cells but also in WS cells in random culture. The MW of 3 min pulse-labeled DNA was slightly larger in WS cells than in normal cells. From the data of the MW of newly synthesized DNA labeled with ^{3}H-thymidine with chasing 0 to 30 min the velocity of DNA elongation to several (2 or 3) replicons level could be estimated. As can be seen in Fig. 3, DNA elongation rate to several replicons does not differ in WS cells comparing with in normal cells. Among normal cells of early passage from variously aged donors, no difference was observed in DNA elongation rate to several replicons.

TABLE 2. THE DURATION OF THE INTERNAL PERIODS OF THE CELL CYCLE AND LABELING INDEX.

Cells	T_{G1}* (h)	T_{G2}** (h)	T_S† (h)	T_C§ (h)	L.I.‖ (%)
N 1	5.8	4.8	8.4	19.0	37.8
N 2	4.8	5.1	8.9	18.8	36.2
N 3	6.7	4.8	7.4	18.9	32.0
mean	5.8 ± 0.95	4.9 ± 0.17	8.2 ± 0.76	18.9 ± 0.10	35.3 ± 3.0
WS 1	6.3	4.7	11.4	22.4	26.7
WS 2	5.2	5.3	10.5	21.0	17.6
WS 3	5.5	5.1	10.5	21.1	21.5
WS 4	5.0	5.1	10.9	21.0	29.8
WS 5	4.7	4.6	11.1	20.4	29.5
mean	5.3 ± 0.61	5.0 ± 0.30	10.9 ± 0.39	21.2 ± 0.74	25.0 ± 5.3

*T_{G1}: duration of G1 phase + 0.5 mitosis
*T_{G2}: duration of G2 phase + 0.5 mitosis
†T_S: duration of S phase
§T_C: duration of entire cell cycle
‖L.I.: labeling index

DISCUSSION

In this communication, we analyzed the cell cycle of WS cells comparing it with that of normal cells. The results indicate that S phase is specifically prolonged in WS cells in comparison with normal cells and, as a result, whole cell cycle time is prolonged. In the case of

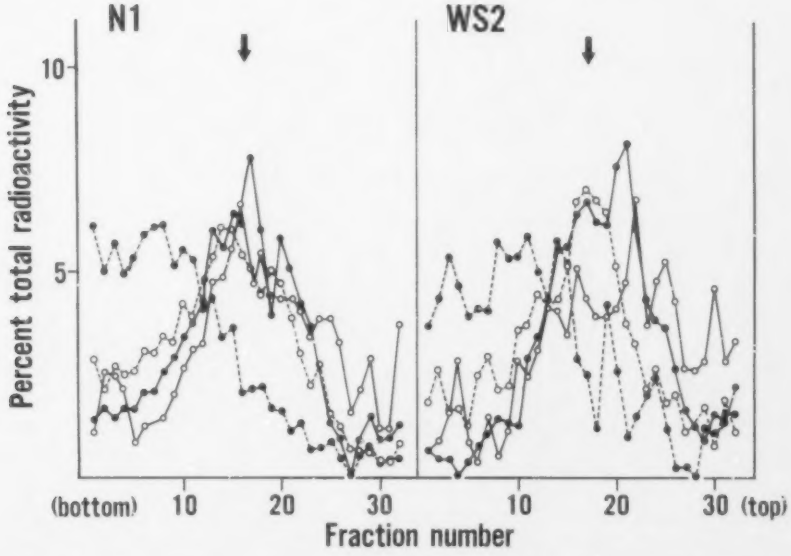

FIG. 2. Alkaline sucrose sedimentation profiles of pulse-labeled and chased DNA from normal and Werner's syndrome cells. Only typical cases are shown (N1 and WS2). Methods were described in the text, in detail. Arrows indicate the peak position of T4D DNA.

——○—— 3 min pulse
——●—— 3 min pulse and 10 min chase
--------○-------- 3 min pulse and 30 min chase
--------●-------- 3 min pulse and 120 min chase

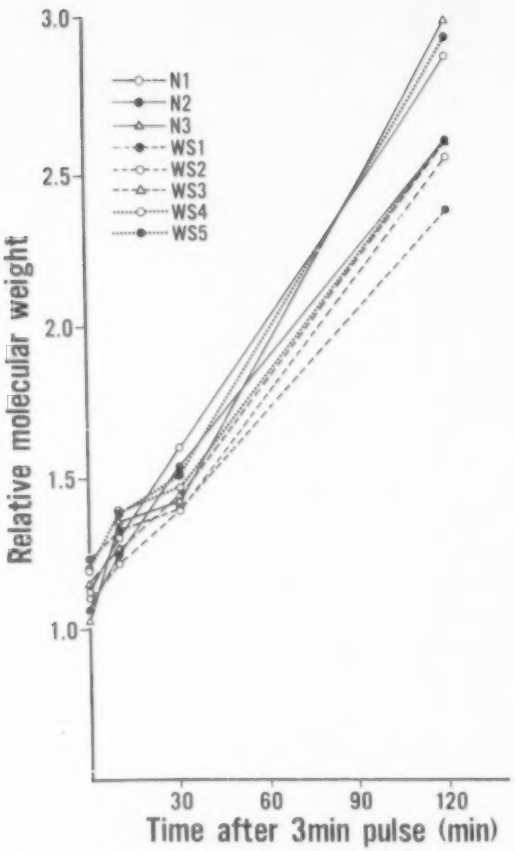

FIG. 3. The mean molecular weights of labeled DNA from three normal cells and five WS cells for different chasing time. The mean values were calculated from the formula of Abelson and Thomas (1966).

normal fibroblasts in culture, the prolongation of cell cycle duration in late passage is primarily due to changes in G1 phase (Yanishevsky *et al.*, 1974; Grove and Cristofalo, 1977) and partly due to changes in G2 phase (Macieira-Coelho *et al.*, 1966; Grove and Cristofalo, 1977). WS cells are generally considered to be an excellent model for human "aging," however, these indicate that WS cells are somewhat different from normal human fibroblasts in late passage.

As for the cause of the prolongation of S phase, two main reasons are conceivable. For the first, slower rate of DNA chain elongation could decrease the overall rate of DNA replication. Slowing rate of DNA chain elongation has been reported in Bloom's syndrome fibroblasts (Hand and German, 1975, Ockey, 1979) and human basal cell carcinoma (Heenen and Galand, 1980). In cultured fibroblasts from human fetal lung, slower rate of DNA chain growth in senescent culture than in nonsenescent culture has also been described (Petes *et al.*, 1974). In WS cells, Fujiwara *et al.* (1977) reported that cultured cells derived from some WS patients showed slower rate of DNA elongation, but

other WS cells had almost normal rate of DNA chain growth by alkaline sucrose gradient centrifugation. They suggested this as a reflection of the heterogeneity of this syndrome. In our alkaline sucrose sedimentation study, no clear difference of DNA elongation rate was observed between normal and WS cells. Since we chased 3 min pulse-labeled DNA for at most 120 min, and this 120 min chased DNA has reached the size of bulk DNA (2–3 replicon size) as calculated from the size of T4D marker DNA and the average replicon size (Huberman and Riggs, 1968), it is difficult to know whether there is a change in the maturation step of these 2–3 replicon-sized DNA. Even if change in the higher level of DNA elongation existed in WS cells, the cause of it would not be a defect in DNA ligase, because the lower level of DNA elongation (that is, joining of Okazaki pieces) also needs DNA ligase and our data show no difference between WS and normal cells in this step. Change in the chromatin structure is more attractive and probable.

Secondly, decrease in the frequency of the replicon initiation could be the cause of the prolongation of S phase. It has been demonstrated that replicon size is not uniform in eukaryotic cells (Hand, 1978), and many potential initiation sites are present at 4 μm intervals along the chromosome (Taylor, 1977). For example, Hand and Tamm reported that the distance between initiation sites labeled during 10 min pulse was increased in mouse fibroblasts infected with reovirus (Hand and Tamm, 1974). Conversely the decrease of the replicon size was reported in SV40-transformed Chinese hamster lung cells (Martin and Oppenheim, 1977). In our preliminary experiments using DNA fiber autoradiography, decrease of the frequency of replicon initiation was observed (Takeuchi *et al.*, 1982). Simply thinking, prolongation of S phase only could cause the increase of labeling index. In WS cell, however, labeling index decreased on the contrary (Tanaka *et al.*, 1979 and this report). This indicates that there are at least two kinds of abnormalities in the cell cycle of WS cells; one is the prolongation of S phase and the other is the increase of the proportion of non-cycling cells. We do not have any data about the cause of the decreasing proportion of cycling cells in WS cells. Recently, a chromosome instability in WS cells has been reported (Salk *et al.*, 1981b), and this could be a cause of the reduced growth. In any event, these two abnormalities would cause the decrease of population growth rate (Salk *et al.*, 1981a), and the decrease of overall rate of DNA replication (Fujiwara *et al.*, 1977) in WS cells.

Finally, presence of a senescent factor(s) in WS cells which may suppress DNA synthesis of WS cells is presumed (Tanaka *et al.*, 1979, 1980; Salk *et al.*, 1981a) by cell hybridization study. It remains unclear how this factor takes part in the prolongation of S phase and the decrease of labeling index in WS cells. In any way, these two characteristics might be useful for the early diagnosis of this genetic disease.

SUMMARY

The duration of the interphase periods of the cell cycle was determined by the percent labeled mitosis method in early passage fibroblasts from five Werner's syndrome and three normal subjects. The average cell cycle time was prolonged in Werner's syndrome cells comparing it to normal cells due to changes in the duration of S phase. The durations of G1 and G2 were not different. In addition, labeling index was decreased in Werner's syndrome cells, suggesting that two abnormal mechanisms in cell cycle existed in Werner's syndrome cells. Using alkaline sucrose gradient sedimentation, the rate of DNA elongation up to 2–3 replicons was examined, and no difference was observed between Werner's

syndrome cells and normal cells. In normal cells in early passage, donor age did not affect the interphase periods of cell cycle and DNA elongation rate up to 2–3 replicons.

Note—Dr. H. Shimada of Tokyo Metropolitan Geriatric Hospital have examined the cell cycle time of a WS strain, and also found the prolongation of S phase in the WS cells (personal communication).

Acknowledgement—This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, and Life Science Grant from the Institute of Physical and Chemical Research, Tokyo, Japan. We thank Drs. T. Matsumura, Y. Horiuchi, K. Murata, A. Hashimoto, Y. Nishida, T. Chihara, K. Ishihara and N. Hasegawa for kind advices. We also thank Dr. H. Ikeda for the kind supply of T4D phage.

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Experimental Gerontology, Vol. 17, pp. 481–487, 1982
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0531-5565/82/060481-07$03.00/0
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LIPOFUSCIN PIGMENT ACCUMULATION IN HUMAN BRAIN DURING AGING

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(*Received* 3 *March* 1982)

Abstract—Histopathologic and autofluorescence investigations were carried out to study lipofuscin pigment accumulation in various age groups of human beings died in accidents (in 100 cases ranging from 1–70 years-of-age). Lipofuscin pigment granules were first observed to appear at 9 years of age. Qualitative studies revealed a progressive increase in the intracellular lipofuscin pigment accumulation with advancing age. These pigment granules were found to congregate in mass in nerve cells of old individuals. An increase in the lipofuscin pigment accumulation and decrease of Nissl substance was observed during aging. The percentage of pigmented nerve cells and the percentage of cytoplasmic area occupied by lipofuscin pigment granules increased significantly with the advancement of age.

INTRODUCTION

THE IMPORTANT characteristic of mammalian nervous system is that the nerve cells do not divide after reaching final state of differentiation during early postnatal life in comparison to those where cellular replacement remains continuous by mitosis throughout infancy, maturity and old age. So, obviously the cause of aging may be linked directly with the loss of these post mitotic nerve cells. Brody (1970) examined superior frontal gyrus of eighteen human samples and reported that nerve cells decreased to about half in old age.

Lipofuscin pigment accumulation has been reported as one of the most important neurocytological change occurring with age (Brody, 1960; Samorajski *et al.*, 1964, 1965, 1968; Whiteford and Getty, 1966; Few and Getty, 1967, Nanda and Getty, 1971, 1973 and Brizzee *et al.*, 1974, 1976). Tappel *et al.* (1973) did not notice any significant increase of lipofuscin pigment in nerve cells of mouse with advancing age. There are very few quantitative studies on lipofuscin pigment accumulation (Reichel *et al.*, 1969 and Brizzee *et al.*, 1974) and no significant report exists on intraneuronal lipofuscin pigment accumulation in nerve cells of cerebral cortex of man in different age groups.

There are contradictory reports regarding the onset of lipofuscin pigment in the nerve cells. Lipofuscin pigment has been observed to appear in the nerve cells of Children (Obersteiner, 1903 and Chu, 1954) and fetus (Humphrey, 1944). While Brody (1960) denied the presence of lipofuscin pigment in the new born. Later on, Pallis *et al.* (1967) reported the absence of pigment in nerve cells of persons below 10 years of age.

The survey of existing literature indicates that the studies on changes with age in human

brain of different age groups are inadequate. Moreover, no attempt has been made to study changes with age in Indians. The present study has been undertaken to examine age changes in human brain of different age groups with a view:

(1) to determine the earliest onset of lipofuscin pigment in nerve cells of superior frontal gyrus;

(2) to determine an increase in the percentage of pigmented nerve cells with age;

(3) to find out an increase in the percentage of cytoplasmic area occupied by lipofuscin pigments with the advancement of age; and

(4) to find out a link in the accumulation of lipofuscin pigment and a decrease of Nissl substance during aging.

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MATERIALS AND METHODS

The human post-mortem brain tissue (from 100 accidental cases ranging from 1–70 years-of-age) was collected from the police morgue of N.R.S. Medical College, Calcutta, India. Every care was taken to get the material from healthy normal persons. The superior frontal gyrus from frontal cerebral cortex was immediately removed after the autopsy and fixed in 10% neutral buffered formalin within 24 hours after death. The fixed tissues were washed, dehydrated, cleared and embedded in paraffin. Paraffin sections were cut at a thickness of 4 μ. The unstained sections were examined under blue light fluorescence microscope. For histologic and histochemical studies, the sections were stained with Sudan black B, Oil red 'O', Long Ziehi Neelsen carbol fuscin, and Schmorl methods.

For the estimation of percentage of neurons containing pigment granules, the nerve cells were randomly selected from Sudan black B stained sections and examined in the light microscope. The percentage of nerve cells that contained pigment granules was obtained by an examination of 100 cells from each slide.

The percentage of cytoplasmic areas occupied by lipofuscin pigment granules was measured by drawing randomly twenty camera lucida diagrams from each Sudan black B stained slide on mm² graph paper under oil immersion field of the microscope. The percentage of area occupied by pigment granules in the cytoplasm was then calculated.

OBSERVATIONS

Histopathologic and autofluorescence investigations were carried out to study qualitative as well as quantitative intracellular lipofuscin pigment accumulation with advancing age.

Qualitative observations

Autofluorescence and histological examination revealed the absence of lipofuscin pigment in the nerve cells of young children. Lipofuscin pigment granules first observed in the cytoplasm of nerve cells at 9 years-of-age. These pigment granules were observed to accumulate progressively in the nerve cells with advancing age. In the second decade, the number of pigment granules increased more than that observed at 9 years-of-age. By the third decade, more pigment bodies appeared and became prominent. In the fourth decade, the pigment bodies were more closely associated near the nuclear periphery. During the fifth decade, lipofuscin pigment accumulation was intense and occupied a greater part of the cytoplasm. Similarly in the sixth and seventh decades, the pigment accumulation was intense and became concentrated in masses, and in some neurons 50% of the cytoplasmic area was occupied by these pigments. It was also observed that the whole of the nuclear periphery in some nerve cells was surrounded by accumulated lipofuscins.

Lipofuscin pigment granules appeared light yellow in young persons and intense golden yellow in old individuals when unstained sections were examined under ultraviolet light. These pigments were insoluble in lipid solvents as the pigments were stainable by various histochemical stains in paraffin sections. These pigments were positive to lipid stains like Sudan black B and Oil red 'O'. Lipofuscins were stained by alcian blue/periodic acid Schiff reaction which revealed their carbohydrate nature. The proteinous nature of the pigments was revealed by their positive staining with mercury bromphencl blue. These pigments were observed to be acid fast and positive to Schmorl reaction. When the sections were examined under ultraviolet light, the fluorescence corresponded only to those areas which were positive to histological reactions in the same series of sections.

In young persons, Nissl substance was seen distributed in the cytoplasm. With the advancement of age, some changes were observed that Nissl granules were pushed towards the cellular periphery during aging since lipofuscin pigments appeared and accumulated above the nucleus. A little RNA or Nissl substance was seen in the nerve cells in which more cytoplasmic area was occupied by lipofuscins in comparison to those cells which contained a few pigment granules or were free from lipofuscins.

Quantitative observations

The quantitative increase in the intracellular lipofuscin accumulation was estimated by the increase in the percentage of nerve cells that contained lipofuscin pigment granules and the percentage of cytoplasmic area occupied by lipofuscin pigment granules in six age decades, i.e., from 11–70 year-old individuals. The percentage of nerve cells that contained pigment granules increased progressively with advancing age (Table 1; Fig. 1). The differences in the percentage of pigmented nerve cells between the second decade and third decade onwards were significant ($p < 0.001$).

The percentage of cytoplasmic pigmented area increased progressively with advancing age (Table 2; Fig. 2). The differences in the percentage of cytoplasmic pigmented area between the second and the sixth decade, and second and the seventh decade were significant ($p < 0.001$).

TABLE 1. CHANGES WITH AGE IN THE PERCENTAGE OF NERVE CELLS THAT CONTAIN LIPOFUSCIN PIGMENT GRANULES.

Age Group	Age-in-Years	Number of subjects	Percentage of pigmented nerve cells
A	11–20	9	32.33 ± 2.05
B	21–30	11	44.81 ± 2.17
C	31–40	12	48.42 ± 1.98
D	41–50	10	51.80 ± 1.86
E	51–60	15	57.53 ± 1.87
F	61–70	15	68.47 ± 2.06

This table does not include persons below 11 years-of-age as the pigment started appearing at 9 years-of-age.

$p < 0.001$ A and B, A and C, A and D, A and E, A and F, B and E, B and F, C and F, D and F, E and F.

$p < 0.01$ C and E.

$p < 0.05$ B and D, D and E.

NS B and C, C and D.

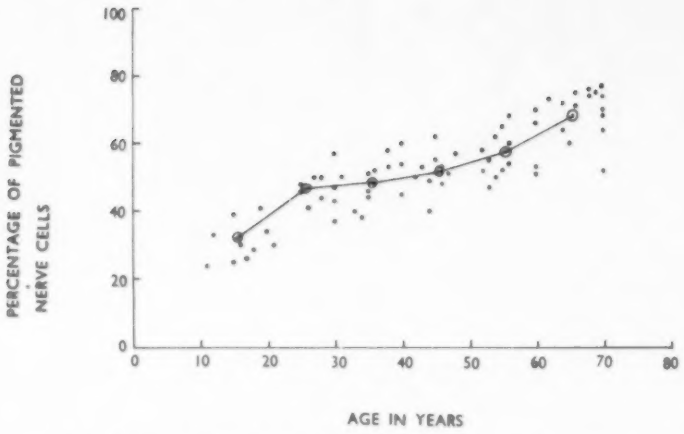

FIG. 1. Changes with age in the percentage of pigmented nerve cells.

DISCUSSION

A number of workers considered lipofuscin accumulation as the most important neurocytological change with age (Brody, 1960; Samorajski, *et al.*, 1964, 1965, 1968; Whiteford and Getty, 1966, Few and Getty, 1967; Nanda and Getty, 1971, 1973; Brizzee *et al.*, 1974, 1975, 1976). The present study revealed a constant accumulation of lipofuscin pigments in nerve cells with age. A significant increase in the percentage of pigmented nerve cells and the percentage of cytoplasmic area occupied by lipofuscin pigment granules was also observed during aging (Tables 1, 2; Figs. 1, 2). Samorajski *et al.* (1968) observed an increase in the percentage of cytoplasmic area occupied by lipofuscin pigment granules and percentage of pigmented nerve cells in mice with advancing age.

The earliest appearance of lipofuscin pigment in man was observed at different ages by

TABLE 2. CHANGES WITH AGE IN THE PERCENTAGE OF AREA OCCUPIED BY LIPOFUSCIN PIGMENT GRANULES IN THE CYTOPLASM OF NERVE CELLS

Age Group	Age-in-Years	Number of subjects	Percentage of area occupied by lipofuscin pigment bodies in the cytoplasm
A	11–20	9	9.25 ± 1.17
B	21–30	11	13.07 ± 0.77
C	31–40	12	13.97 ± 0.86
D	41–50	10	15.44 ± 1.16
E	51–60	15	18.42 ± 0.86
F	61–70	15	21.08 ± 0.95

This table does not include persons below 11 years of age as the onset of pigment started at 9 years of age.

$p < 0.001$ A and E, A and F, B and E, B and F, C and E, C and F.

$p < 0.01$ A and C, A and D, D and F.

$p < 0.05$ A and B, E and F.

NS B and C, B and D, C and D, C and E.

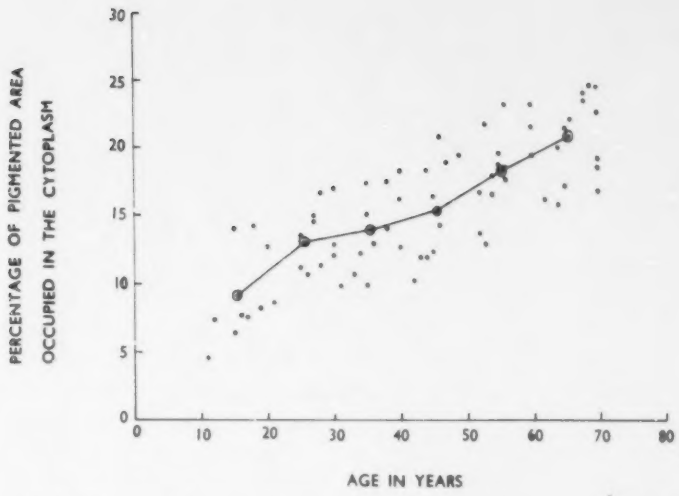

FIG. 2. Changes with age in the percentage of pigmented area occupied in the cytoplasm of nerve cells.

various workers. The pigments were observed in the spinal cord of human fetus by Humphrey (1944). These pigments were not observed in the nerve cells of the new born (Brody, 1960). Lipofuscin pigments were observed in the motor neurons of lumbar spinal cortex in children by Obersteiner (1903) and Chu (1954). Pallis *et al.* (1967) reported absence of pigment in normal persons below 10 years-of-age. In the present study, however, lipofuscin pigments were observed as early as 9 years-of-age.

Mühlmann (1910) observed that lipofuscin pigment granules, which were distributed homogenously, started accumulating in clusters and finally localized in mass that continued to increase in size with age. The pigment was found to appear in a congregate mass in a polarized pattern deep to the base of the apical dendrites in pyramidal neurons of motor cortex in rat and pyramidal layer of hippocampus in rat and monkey (Brizzee *et al.*, 1969, 1974; and Brizzee and Johnson, 1970). Whiteford and Getty (1966) described four types of distribution patterns of lipofuscins (homogenous, perinuclear, polar and bipolar) in nerve cells of dog. Nanda and Getty (1971) and Nandy (1971) described their appearance at the perinuclear position. The present study showed the early appearance of lipofuscin pigment at the perinuclear position. Thereafter, lipofuscin pigment granules increased with age and started accumulating at the perinuclear position. Later on, these pigments became concentrated in mass which continued to increase in size at the nuclear periphery in old individuals.

Hydén and Lindström (1950), Pope and Hess (1957) and Mann and Yates (1974) reported that during cellular aging, Nissl substance decreased whereas pigment amount increased. It was found in the present study that Nissl substance was pushed towards the cellular periphery in nerve cells of old individuals as the pigment accumulated at the nuclear periphery. Nissl substance was observed in small quantity in nerve cells which were occupied by more lipofuscins in comparison to those which contained a very small quantity of lipofuscin pigment granules.

The present study showed that lipofuscin pigments were positive to histochemical tests

for lipids, glycogens, proteins, acid fast groups and Schmorl reaction. Strehler (1964) also reported that these pigments were positive to similar kinds of histochemical reactions.

As the nerve cells do not divide, their loss in the aging nervous system may be associated with intraneuronal lipofuscin accumulation. Samorajski *et al.* (1968), Zeman (1974) and Brizzee et al. (1975) suggested that intraneuronal lipofuscin accumulation during aging may cause loss of nerve cells. But Brizzee *et al.* (1968) reported an increase of nerve cells in rat with the advancement of age. No loss of nerve cells was reported in guinea pig (Wilcox, 1956), house fly (Sohal and Sharma, 1972). A considerable loss of nerve cells was reported in mice (Johnson and Erner, 1972) and man (Brody, 1955, 1970 and Ball, 1977). Brody (1970) reported 50% cell loss in superior frontal gyrus with advancing age. The present study showed a considerable accumulation of lipofuscin pigment and decrease of Nissl substance in nerve cells of superior frontal gyrus during aging. A constant and progressive accumulation of lipofuscin pigment in nerve cells indicates an aging process. As in the present study, a considerable accumulation of lipofuscin pigment and a decrease of Nissl substance has been observed with age, it seems quite reasonable to assume that the presence of pigment in nerve cells occupying most of the cytoplasmic area may be detrimental to normal cellular metabolism. The physiology and metabolic functions of nerve cells may probably be affecting because of reduction in cytoplasmic area in old age. The reduction in RNA content or Nissl substance of cell may be such that the cell is unable to maintain its metabolic activity and cell death or atrophy may occur.

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